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APPLICATION NUMBER: *60/484,923*
FILING DATE: *July 03, 2003*

DK/04/478

PRIORITY DOCUMENT
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DOCKET NUMBER
Jensen et al.
09663.0066USP1

CERTIFICATE UNDER 37 CFR 1.10:
"Express Mail" mailing label number: EV322885580US
Date of Deposit: July 3, 2003

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By: _____
Name: Jack Junker

REQUEST FOR PROVISIONAL APPLICATION UNDER 37 C.F.R. § 1.53(c)

MAIL STOP PROVISIONAL PATENT APPLICATION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This is a request for filing a Provisional application for patent under 37 CFR § 1.53(c) entitled NOD-FACTOR PERCEPTION by the following inventor(s):

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1. ☒ Enclosed is the Provisional application for patent as follows: 128 pages of specification, and 13 sheets of drawings.

2. ☐ Small entity status is claimed pursuant to 37 CFR 1.27.

3. ☒ Payment of Provisional filing fee under 37 C.F.R. § 1.16(k) :
   - ☒ Attached is a check in the amount of $ 160.00.
   - ☐ Please charge Deposit Account No. 13-2725.
   - ☐ PAYMENT OF THE FILING FEE IS BEING DEFERRED.

4. ☒ The Commissioner is hereby authorized to charge any additional fees as set forth in 37 CFR §§ 1.16 to 1.18 which may be required by this paper or credit any overpayment to Account No. 13-2725.

5. ☐ Enclosed is an Assignment of the invention to , Recordation Form Cover Sheet and a check for $ to cover the Recordation Fee.

6. ☒ Also Enclosed: Application Data Sheet 5 Pages

7. ☐ The invention was made by the following agency of the United States Government or under a contract with the following agency of the United States Government:

8. ☒ Address all future communications to the **Attention of Denise Kettelberger** (may only be completed by attorney or agent of record) at the address below.

9. ☒ A return postcard is enclosed.

Respectfully submitted,

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Date: 3 July 2003

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Application Data Sheet

Application Information

Application Type::	Provisional
Subject Matter::	Utility
Suggested Classification::	
Suggested Group Art Unit::	
CD-ROM or CD_R?::	None
Number of CD disks::	
Number of copies of CDs::	
Sequence Submission::	No
Computer Readable Form (CRF)?::	No
Title::	NOD-FACTOR PERCEPTION
Attorney Docket Number::	09663.0066USP1
Request For Early Publication::	No
Request For Non-Publication::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	13
Small Entity::	No
Latin Name::	
Variety Denomination Name::	
Petition Included::	No
Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers::	
Secrecy Order in Parent Appl.?::	No

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Initial 07/03/03

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Initial 07/03/03

Nod-factor perc ption

Field of the invention

The invention relates to a novel Nod-factor binding element and component polypeptides that are useful in enhancing Nod-factor binding in nodulating plants and inducing nodulation in non-nodulating plants. More specifically, the invention relates to Nod-factor binding proteins and their respective genomic and mRNA nucleic acid sequences.

Background of the invention

The growth of agricultural crops is almost always limited by the availability of nitrogen, and at least 50% of global needs are met by the application of synthetic fertilisers in the form of ammonia, nitrate or urea. Apart from recycling of crop residues and animal manure, and atmospheric deposition, the other most important source of nitrogen for agriculture comes from biological nitrogen fixation.

A small percentage of prokaryots, the diazotrophs, produce nitrogenases and are capable of nitrogen fixation. Members of this group, belonging to the *Rhizobiaceae* family (for example *Mesorhizobium loti*, *Rhizobium meliloti*, *Bradyrhizobium japonicum*, *Rhizobium leguminosarum* bv *viceae*) here collectively called *Rhizobium* or *Rhizobia* spp and the actinobacterium *Frankia* spp, can form endosymbiotic associations with plants conferring the ability to fix nitrogen. Although many plants can associate with nitrogen fixing bacteria, only a few plants, all members of the Rosid I Clade, form endosymbiotic associations with *Rhizobia* spp and *Frankia* spp., which are unique in that most of the nitrogen is transferred to and assimilated by the host plant. Legumes, including soybean, bean, pea, peanut, chickpea, cowpea, lentil, pigeonpea, alfalfa and clover, are the most agronomically important members of this small group of nitrogen-fixing plants.

The rhizobial-legume interaction is generally host-strain specific, whereby successful symbiotic associations only occur between specific rhizobial

strains and a limited number of legume species. The specificity of this interaction is determined by chemical signalling between plant and bacteria, which accompanies the initial interaction and the establishment of the symbiotic association (Hirsch *et al.* 2001, *Plant Physiol.* 127: 1484-1492).

Specific (iso)flavanoids, secreted into the soil by legume spp, allow *Rhizobium* spp to distinguish compatible hosts in their proximity and to migrate and associate with roots of the host. In a compatible interaction, the (iso)flavanoid perceived by the *Rhizobium* spp, interacts with the rhizobial *nodD* gene product, which in turn leads to the induction of rhizobial Nod-factor synthesis. Nod-factor molecules are lipo-chitin-oligosaccharides, commonly comprising four or five β-1-4 linked N-acetylglucosamines, with a 16 to 18 carbon chain fatty acid n-acetylated on the terminal non-reducing sugar. Nod factors are synthesised in a number of variants, characterised by their chemically different substitutions on the chitin backbone which are distinguished by the compatible host plant. The perception of Nod-factors by the host induces invasion zone root hairs, in the proximity of rhizobial cells, to curl and entrap the bacteria. The adjacent region of the root hair plasma membrane invaginates and new cell wall material is synthesized to form an infection thread or tube, which serves to transport the symbiotic bacteria through the epidermis to the cortical cells of the root. Here the cortical cells are induced to divide to form a primordium, from which a root nodule subsequently develops. In legumes belonging to genera like *Arachis* (peanut), *Stylosantos* and *Sesbania,* infection is initiated by a simple "crack entry" through spaces or cavities between epidermal cells and lateral roots.

In spite of these differences, perception of Nod factors by the host plant simultaneously induces the expression of a series of plant nodulin genes, which control the development and function of root nodules, wherein the rhizobial endosymbiotic association and nitrogen fixation are localised.

A variety of molecular approaches have identified a series of plant nodulin genes which play a role in rhizobial-legume symbiosis, and whose expression is induced at early or later stages of rhizobial infection and nodule

development (Geurts and Bisseling, 2002, *Plant Cell* supplement S239-249). Furthermore, plant mutant studies have revealed that a signalling pathway must be involved in amplifying and transducing the signal resulting from nod-factor perception, which is required for the induction of nodulin gene expression. Among the first physiological events identified in this signal transduction pathway, which occurs circa 1 min after Nod-factor application to the root epidermis, is a rapid calcium influx followed by chloride efflux, causing depolarisation of the plasma membrane and alkalization of the external root hair space of the invasion zone. A subsequent efflux of potassium ions allows re-polarisation of the membrane, and later a series of calcium oscillations are seen to propagate the signal through the root hair cell. Pharmacological studies with specific drugs, which mimic or block Nod-factor induced responses, have identified potential components of the signalling pathway. Thus mastoparan, a peptide which is thought to mimic the activated intracellular domain of G-protein coupled receptors, can induce early Nod gene expression and root hair curling. This suggests that trimeric G protein may be involved in the Nod-factor signal transduction pathway. Analysis of a group of nodulation mutants, including some that fail to show calcium oscillations in response to Nod-factor signals, has revealed that in addition to the lack of nodulation, these mutants are unable to form endosymbioses with arbuscular mycorrhizal fungi. This implies that a common symbiotic signal transduction pathway is shared by two types of endosymbiotic relationships, namely root nodule symbiosis, which is largely restricted to the legume family, and arbuscular mycorrhizal symbiosis, which is common to the majority of land plant species. This suggests that there may be a few key genes which dispose legumes to engage in nodulation, and which are missing from crop plants such as cereals.

The identification of these key genes, which encode functions which are indispensable for establishing a nitrogen fixing system in legumes, and their transfer and expression in non-nodulating plants, has long been a goal of molecular plant breeders. This could have a significant agronomic impact on

the cultivation of cereals such as rice, where production of two harvests a year may require fertilisation with up to 400 kg nitrogen per hectare. In accordance with this goal, WO02102841 describes the gene encoding the NORK polypeptide, isolated from the nodulating legume *Medicago sativa*, and the transformation of this gene into plants incapable of nitrogen fixation. The NORK polypeptide and its homologue/orthologue SYMRK from *Lotus japonicus* (Stracke *et al* 2002 *Nature* 417:959-962), are transmembrane receptor-like kinases with an extracellular domain comprising leucine-rich repeats, and an intracellular protein kinase domain. *Lotus japonicus* mutants, with a non-functional *SYMRK* gene, fail to form symbiotic relationships with either nodulating rhizobia or arbuscular mycorrhiza. This implies that a common symbiotic signalling pathway mediates these two symbiotic relationships, where SYMRK comprises an early step in the pathway. The *symRK* mutants retain an initial response to rhizobial infection, whereby the root hairs in the susceptable invasion zone undergo swelling of the root hair tip and branching, but fail to curl. This suggests that the SYMRK protein is required for an early step in the common symbiotic signalling pathway, located downstream of the perception and binding of microbial signal molecules (e.g. Nod-factors), that leads to the activation of nodulin gene expression.

The search for key symbiosis genes has also focussed on 'candidate genes' encoding receptor proteins with the potential for perceiving and binding Nod-factors or surface structures on rhizobial bacteria. US 6,465,716 discloses NBP46, a Nod-factor binding lectin isolated from *Dolichos biflorus* roots, and its transgenic expression in transformed plants. Transgenic expression of NBP46 in plants is reported to confer the ability to bind to specific carbohydrates in the rhizobial cell wall and thereby to bind these bacteria and utilise atmospheric nitrogen, as well as conferring apyrase activity. An alternative approach to search for key symbiosis genes has been to screen for Nod-factor binding proteins in protein extracts of plant roots. NFBS1 and NFBS2 were isolated from *Medicago trunculata* and shown to bind Nod-

factors in nanomolar concentrations, however, they both failed to exhibit the Nod-factor specificity characteristic of rhizobial-legume interactions (Geurts and Bisseling, 2002 *supra*).

The Nod-factor binding element, which is responsible for strain specific Nod-factor perception is not, as yet, identified. The isolation and characterisation of this element and its respective gene(s) would open the way to introducing Nod-factor recognition into non-nodulating plants and thereby the potential to establish *Rhizobium*-based nitrogen fixation in important crop plants.

Rhizobial strains produce strain-specific Nod-factors, lipochitin oligosaccharides (LCOs), which are required for a host-specific interaction with their respective legume hosts. *Lotus* and peas belong to two different cross-inoculation groups, where *Lotus* develops nodules after infection with *Mesorhizobium loti,* while pea develops nodules with *Rhizobium leguminosarum* bv *viceae*. Cultivars belonging to a given *Lotus* sp also vary in their ability to interact and form nodules with a given rhizobial strain. Perception of Nod-factor secreted by *Rhizobium* spp bacteria, as the first step in nodulation, commonly leads to the initiation of tens or even hundreds of rhizobial infection sites in a root. However, the majority of these infections abort and only in a few cases do the rhizobia infect the nodule primordium. The frequency and efficiency of the *Rhizobium*-legume interaction leading to infection is known to be influenced by variations in Nod-factor structure. The genetics of Nod-factor synthesis and modification of their chemical structure in *Rhizobium* spp have been extensively characterised. An understanding of Nod-factor binding and perception, and the structure of its component elements is needed in order to optimise the host Nod-factor response. This information would, in turn, provide the necessary tools to breed for enhanced efficiency of nodulation and nitrogen fixation in current nitrogen-fixing crops.

The importance of this goal is clearly illustrated by the performance of the major US legume crop, soybean, which is grown on 15%, or more, of agricultural land in the US. While nitrogen fixation by soybean root nodules can assimilate as much as 100 kg nitrogen per hectare per year, these high levels of nitrogen assimilation are insufficient to support the growth of the highest yielding modern soybean cultivars, which still require the application of fertiliser.

In summary, there is a need to increase the efficiency of nodulation and nitrogen fixation in current legume crops as well as to transfer this ability to non-nodulating crops in order to meet the nutritional needs of a growing global population, while minimising the future use of nitrogen fertilisers and their associated negative environmental impact.

Summary of the invention

The invention provides a Nod-factor binding element comprising one or more isolated NFR polypeptides. The NFR polypeptides of the invention are NFR1, comprising an amino acid sequence substantially identical to SEQ ID No: 25, having specific Nod-factor binding properties, and NFR5 comprising an amino acid sequence substantially identical to SEQ ID No: 8, having specific Nod-factor binding properties. Furthermore, the invention provides for the isolation of nucleic acid molecules comprising *NFR1* and *NFR5* gene and cDNA sequences encoding said NFR1 polypeptide and said NFR5 polypeptide, comprising a nucleic acid sequence substantially identical to SEQ ID No: 23 and SEQ ID No: 7, respectively.

According to a further embodiment of the invention, a method is provided for producing a plant expressing the Nod-factor binding element, the method comprising introducing into the plant a transgenic expression cassette comprising a nucleic acid sequence, encoding a NFR polypeptide having specific Nod-factor binding properties and having an amino acid sequence substantially identical to SEQ ID No: 25 or SEQ ID No:8, respectively, wherein the nucleic acid sequence is operably linked to its own promoter or a

heterologous promoter, preferably a root specific promoter. In a preferred embodiment, the expression of both said NFR 1 and NFR5 polypeptides by the transgenic plant confers on the plant the ability to bind Nod-factors in a chemically specific manner and thereby initiate the establishment of a *Rhizobium*-plant interaction leading to the development of nitrogen-fixing root nodules.

According to a further embodiment, the invention provides a method for marker assisted breeding of *NFR* alleles, encoding variant NFR polypeptides, comprising the steps of identifying variant NFR1 or NFR5 polypeptides in a nodulating legume species, comprising an amino acid sequence substantially similar to SEQ ID No: 25 or SEQ ID No: 8 respectively; determining the nodulation frequency of plants expressing said variant NRF1 or NFR5 polypeptide; identifying DNA polymorphisms at loci genetically linked to or within the allele locus encoding said variant NFR1 or NFR5 locus; preparing molecular markers based on said DNA polymorphisms; and using said molecular markers for the identification and selection of plants carrying *NFR* alleles encoding said variant NFR1 or NFR5 polypeptides. The invention includes plants selected by the use of this method of marker assisted breeding. In a preferred embodiment, said method of marker assisted breeding of *NFR* alleles provides for the breeding legumes with enhanced nodulation frequency and nodule occupancy.

Brief Description of the figures

Figure 1: Map based cloning of *Lotus NFR5*. **a.** Genetic map of the *NFR5* region with positions of linked AFLP and microsatellite markers above the line and distances in cM below. The fraction of recombinant plants detected in the mapping population is indicated. **b.** Physical map of the BAC and TAC clones between the closest linked microsatellite markers. The positions of sequence-derived markers used to fine-map the *NFR5* locus, and the fraction of recombinant plants found in the mapping population are indicated. **c.** Candidate genes identified in the sequenced region delimited by the closest

linked recombination events. **d.** Structure of the *NFR5* gene, position of the transcription initiation point and the *nfr5-1*, *nfr5-2* and *nfr5-3* mutations. The asterisk indicates the position of a stop codon in *nfr5-3*; the black triangle a retrotransposon insertion in *nfr5-2*; and the grey box defines the deletion in *nfr5-1*. GGDP: geranylgeranyl diphosphate synthase; RE: retroelement; RZF: ring zinc finger protein; GT: glycosyl transferase; A2L: apetala2-like protein; RLK: receptor-like kinase; PL: pectate lyase-like protein; AS: ATPase-subunit; HD: homeodomain protein; RF: ring finger protein. Hypothetical proteins are not labelled. **e.** Southern hybridization demonstrating deletion of SYM10 in the "N15" *sym10* mutant line. *Eco*RI digested genomic DNA of the parental variety "Sparkle" and the fast neutron derived mutant "N15" hybridized with a pea *SYM10* probe covering the region encoding the predicted extracellular domain. Hybridization with a probe from the 3'untranslated region demonstrated that the complete gene was deleted.

Figure 2: Structure and domains of the NFR5 protein. **a.** Schematic representation of the NFR5 protein domains. **b.** The amino acid sequence of NFR5 arranged in protein domains. Bold, conserved LysM residues. Bold and underlined residues conserved in protein kinase domains (KD); TM: transmembrane, SP: signal peptide. The asterisk indicates a stop codon in the *nfr5-3*; the black triangle a retrotransposon insertion in *nfr5-2* and the grey box defines the amino acids deleted in *nfr5-1*. **c.** Individual alignment of the three LysM motifs (M1, M2, M3) from NFR5, pea SYM10, *Medicago truncatula* (*M.t*, Ac126779) rice (Ac103891), the single LysM in chitinase from *Volvox carteri* (Acc. No: T08150) and the pfam consensus. **d.** The divergent or absent activation loop (domain VIII) in the NFR5 family of receptor kinases is illustrated by alignment of kinase motifs VII, VIII and IX from *Arabidopsis* (At2g33580) NFR5, SYM10, *Medicago truncatula* (*M.t*, Ac126779), rice (Ac103891) and the SMART concensus. Conserved domain VII aspartic acid is marked in bold and underlined. **c** and **d** the amino acids conserved in all

aligned motifs are marked in black and amino acids conserved in two or more motifs are marked in grey.

Figure 3. The aligned amino acid sequence of the LjNFR5 and PsSYM10 proteins. Amino acid residues sharing identity are highlighted. The *Medicago truncatula* (Ac126779) showing 76 % amino acid identy to *Lotus* NFR5 is included to exemplify a substantial identical protein sequence.

Figure 4. Steady-state levels of *LjNFR5* and *PsSYM10* mRNA. **a.** *NFR5* mRNA detected in uninoculated roots, inoculated roots, nodules, leaves, flowers and pods of *Lotus* plants. **b.** Time course of *NFR5* mRNA transcript accumulation in roots after inoculation with *M. loti*. The identity of the amplified transcripts was confirmed by sequencing. ATPase was used as internal control and relative normalised values compared to uninoculated roots are shown. **c.** Northern analysis showing *NFR5* mRNA expression in nodule leaf and root of symbiotically and non-symbiotically grown *Lotus* plants. **d.** Northern analysis showing *Sym10* mRNA expression in leaf, root and nodule of symbiotically and non-symbiotically grown pea plants.

Figure 5. Positional cloning of the *NFR1* gene. a. Genetic map of the region surrounding the *NFR1* locus. Positions of the closest AFLP, microsatelitte- and PCR-markers are given together with genetic distances in cM. b. Physical map of the *NFR1* locus. BAC clones 56L2, 16K18, 10M24, 36D15, 56K22 and TAC clones LjT05B16, LjT02D13, LjT211O02, which cover the region are shown. The numbers of recombination events detected with BAC and TAC end-markers or internal markers are given. Arrows indicate the positions of the two markers (10M24-2, 56L2-2) delimiting the *NFR1* locus. UFD and HP correspond to the UFD1-like protein and the hypothetical protein encoded in the region. c. Exon-intron structure of the *NFR1* gene. Boxes correspond to exons, where LysM motifs are shown in light grey, trans-membrane region in black, kinase domains in dark grey. Dotted lines define introns and full lines define the 5' and 3' un-translated regions. The

nucleotide length of all exons and introns are indicated. The numbers between brackets correspond to exon and intron 4, corresponding to alternative splicing.

Figure 6. Structure and domains of the NFR1 protein. **a.** Primary structure of the *NFR1* protein comprising a signal peptide (SP); LysM motifs (LysM1 and LysM2); transmembrane region (TM); protein kinase domains with conserved amino acids in bold and underlined (PK). The cysteine couples (**CxC**) are in bold and the LysM amino acids important for secondary structure maintenance are underlined. The two extra amino acids resulting from alternative splicing are shown in brackets. I-XI represent the kinase domains. Asterisks indicate positions of the nonsense mutations found in *NFR1-1* and *NFR1-2* mutant alleles. **b.** Alignments of the two *NFR1* LysM motifs to the consensus sequences predicted by the SMART program and the *Arabidopsis thaliana* (Acc No: NP566689), rice (*O. sativa*) (Acc No: BAB89226), and *Volvox carteri* (Acc. No: T08150) LysM motifs.

Figure 7. *NFR1*, *NFR5* and *SymRK* gene expression. **a.** Transcript levels of *NFR1* in uninoculated, inoculated roots, nodules, leaves, flowers and pods of wild type plants. **b.** Transcript levels of *NFR1* in wild type, *nfr1, nfr5* and *symRK* mutant plants after inoculation with *M. loti*. **c.** Transcript levels of *NFR5* in wild type, *nfr1, nfr5* and *SymRK* mutant plants after inoculation with *M. loti*. **d.** Transcript levels of *SYMRK* in wild type, *nfr1, nfr5* and *symRK* mutant plants after inoculation with *M. loti*. Transcript levels were measured by quantitative PCR. ATPase was used as internal control and relative values normalised to the untreated roots (zero hours) are shown.

Figure 8. Root hair response after inoculation with *M. loti* or Nod-factor application. **a.** Wild type root hair curling on seedlings inoculated with *M. loti*. **b.** Root hair deformations on wild type seedlings after Nod-factor application. **c.** Root hairs on *nfr1-1* seedlings inoculated with *M. loti*. **d.** Root hairs on

nfr1-1 seedlings after Nod-factor application. **e.** Root hairs with balloon deformations on *symRK-3* mutants inoculated with *M. loti*. **f.** Roots hairs on a *nfr1-1,symRK-3* double mutant inoculated with *M. loti* **g.** Excessive root hair response on *nin* mutants inoculated with *M. loti*. **h.** Root hairs on a *nfr1-1,nin* double mutant inoculated with *M. loti*. Root hairs on *nfr5-1* seedlings inoculated with *M. loti*, *nfr5-1* seedlings after Nod-factor application, untreated *nfr5-1* control, untreated wild type control, untreated *nfr1-1* control, are indistiguisable from the straight roots hairs shown in **c, d, f, h** and therefore not shown. Inserts to the right of **a** to **h** show a close-up of the root hairs.

Figure 9. Membrane depolarisation and pH changes in the extracellular root hair space after application of Nod-factor purified from *M. loti*. Influence of 0.1 µM Nod-factor (NF) on membrane potential (Em) and/or external pH (pH) of **a.** *Lotus* wild type **b.** *nfr5-1* and *nfr5-2* mutants **c.** *nfr1-1* and *nfr1-2* mutants **d.** *symRK-1* and *symRK-3* mutants **e.** *nfr1-2,symRK-3* double mutant, **f.** pH changes in the extracellular root hair space after application of an undecorated chito-octaose.

Figure 10. Expression of the *NIN* and *ENOD2* genes in wild type, *nfr1* and *nfr5* mutant genotypes. **a.** NIN transcript level in RNA extracted from roots two hours to 12 days after *M. loti* inoculation. **b** ENOD2 transcript level in RNA extracted from roots two hours to 12 days after *M. loti* inoculation. Transcript levels were measured by quantitative PCR and the identity of the amplified sequences was confirmed by sequencing. ATPase was used as internal control and relative values normalised to the untreated root (zero hours) are shown.

Figur- 11. Alignment NFR1 and NFR5 proteins reveal an overall similarity of 33 % amino acid identities

Figure 12. Domain structure of native and hybrid NFR1 and NFR5 polypeptides.

Detailed description of the invention

I. Definitions

AFLP: Amplified Fragment Length Polymorphism is a PCR-based technique for the amplification of genomic fragments obtained after digestion with two different enzymes. Different genotypes can be differentiated based on the size of amplified fragments or by the presence or absence of a specific fragment (Vos, P. (1998), *Methods Mol Biol.*, 82:147-155). Amplified Fragment Length Polymorphism is a PCR-based technique used to map genetic loci.

***Agrobacterium rhizogenes*-mediated transformation**: is a technique used to obtain transformed roots by infection with *Agrobacterium rhizogenes*. During the transformation process the bacteria transfers a DNA fragment (T-DNA) from an endogenous plasmid into the plant genome (Stougaard, J. *et al*, (1987) *Mol.Gen.Genet.* 207, 251-255). For transfer of a gene of interest the gene is first inserted into the T-DNA region of *Agrobacterium rhizogenes* which is subsequently used for wound-site infection.

Allele: gene variant

BAC clones: clones from a Bacterial Artificial Chromosome library

Conservatively modified variant: when referring to a polypeptide sequence when compared to a second sequence, includes individual conservative amino acid substitutions as well as individual deletions, or additions of amino acids. Conservative amino acid substitution tables, providing functionally similar amino acids are well known in the art. When referring to nucleic acid sequences, conservative modified variants are those that encode an identical amino acid sequence, in recognition of the fact that codon redundancy allows a large number of different sequences to encode any given protein.

Contig: a series of overlapping cloned sequences e.g. BACs, co-linear and homologous to a region of genomic DNA.

Exons: protein coding sequences of a gene sequence

Expression cassette: refers to a nucleic acid sequence, comprising a promoter operably linked to a second nucleic acid sequence containing an ORF or gene, which in turn is operably linked to a terminator sequence.

Heterologous: A polynucleotide sequence is "heterologous to" an organism or a second polynucleotide sequence if it originates from a foreign species, or from a different gene, or is modified from its original form. A heterologous promoter operably linked to a coding sequence refers to a promoter from a species, different from that from which the coding sequence was derived, or, from a gene, different from that from which the coding sequence was derived.

Homologue: is a gene or protein with substantial identity to another gene's sequence or another protein's sequence.

Identity: refers to two nucleic acid or polypeptide sequences that are the same or have a specified percentage of nucleic acids of amino acids that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using one of the sequence comparison algorithms listed herein, or by manual alignment and visual inspection. When percentage of sequence identity is used in reference to proteins, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to account for the conservative nature of the substitution. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thus increasing the percent identity. Means for making these adjustments are well known to those skilled in the art.

Introns: are non-coding sequences interrupting protein coding sequences within a gene sequence.

LCO: lipochitin oligosaccharides.

Legumes: are members of the plant Family Fabaceae, and include bean, pea, soybean, clover, vetch, alfalfa, peanut, pigion pea, chickpea, fababean, cowpea, lentil in total approximately 20.000 species.

Locus: or "loci" refers to the map position of a nucleic acid sequence or gene on a genome.

Marker assisted breeding: the use of DNA polymorphisms as "molecular markers", (for examples simple sequence repeats (microsatelittes) or single nucleotide polymorphism (SNP)) which are found at loci, genetically linked to, or within, the *NFR1* or *NFR5* loci, to breed for advantageous *NFR* alleles.

Molecular markers: refer to sites of variation at the DNA sequence level in a genome, which commonly do not show themselves in the phenotype, and may be a single nucleotide difference in a gene, or a piece of repetitive DNA.

Monocotyledenous cereal: includes, but is not limited to, barley, maize, oats, rice, rye, sorghum, and wheat.

Mutant: a plant or organism with a modified genome sequence resulting in a phenotype which differs from the common wild-type phenotype.

Native: as in "native promoter" refers to a promoter operably linked to its homologous coding sequence.

NFR : refers to *NFR* genes, in particular *NFR1* and *NFR5* genes which encode NFR1 and NFR5 polypeptides respectively, and comprise a nucleic acid sequence substantially identical to SEQ ID No: 23 and SEQ ID No: 7, respectively.

NFR polypeptides: are polypeptides that are required for Nod-factor binding and function as the Nod-factor binding element in nodulating plants. NFR polypeptides include the NFR5 polypeptide, having an amino acid sequence substantially similar to SEQ ID No: 8, and the NFR1 polypeptide, having an amino acid sequence substantially similar to SEQ ID No: 25. NFR5 and NFR1 polypeptides show little sequence homology, but they share a similar

domain structure comprising an N-terminal signal peptide, an extracellular domain having 2 or 3 LysM-type motifs, followed by a transmembrane domain, followed by an intracellular domain comprising a kinase domain characteristic of serine/threonine kinases. The extracellular domain of NFR proteins is the primary determinant of the specificity of Nod-factor recognition, whereby a host plant comprising a given *NFG* allele will only form nodules with one or a limited number of *Rhizobium* strains.

Northern blot analysis: a technique for the quantitative analysis of mRNA species in an RNA preparation.

Nod-factors: are synthesised by nitrogen-fixing *Rhizobium* bacteria, which form symbiotic relationships with specific host plants. They are lipo-chitin-oligosaccharides (LCOs), commonly comprising four or five β-1-4 linked N-acetylglucosamines, with a 16 to 18 carbon chain fatty acid n-acetylated on the terminal non-reducing sugar. Nod-factors are synthesised in a number of chemically modified forms, which are distinguished by the compatible host plant.

Nod-factor binding element: comprises one or more NFR polypeptides present in the roots of nodulating plants, and functions in detecting the presence of Nod-factors at the root surface and within the root and nodule tissues. The NFR polypeptides, which are essential for Nod-factor detection, comprise the first step in the Nod-factor signalling pathway that triggers the development of an infection thread and root nodules.

Nod-factor binding properties: are a characteristic of NFR1 and NFR5 polypeptides and are particularly associated with the extracellular domain of said NFR polypeptides, which comprise LysM domains. The binding of Nod-factors by the extracellular domain of NFR polypeptides is specific, such the NFR polypeptides can distinguish between the strain-specific chemically modified forms of Nod-factor.

Nodulating plant: a plant capable of establishing an endosymbiotic *Rhizobium* – plant interaction with a nitrogen-fixing *Rhizobium* bacterium, including the formation of an infection thread, and the development of root

nodules capable of fixing nitrogen. Nodulating plants are limited to a few plant families, and are particularly found in the *Legume* family, and they are all member of the Rosid 1 clade.

Non-nodulating plant: a plant which is incapable of establishing an endosymbiotic *Rhizobial* – plant interaction with a nitrogen-fixing *Rhizobial* bacterium, and which does not form root nodules capable of fixing nitrogen.

Operably linked: refers to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates transcription of RNA corresponding to the second sequence.

ORF: Open Reading Frame, which defines one of three putative protein coding sequences in a DNA polynucleotide.

Orthologue: Two homologous genes (or proteins) diverging concurrently with the organism harbouring them diverged. Orthologues commonly serve the same function within the organisms and are most often present in a similar position on the genome.

PCR: Polymerase Chain Reaction is a technique for the amplification of DNA polynucleotides, employing a heat stable DNA polymerase and short oligonucleotide primers, which hybridise to the DNA polynucleotide template in a sequence specific manner and provide the primer for 5' to 3' DNA synthesis. Sequential heating and cooling cycles allow denaturation of the double-stranded DNA template and sequence-specific annealing of the primers, prior to each round of DNA synthesis. PCR is used to amplify DNA polynucleotides employing the following standard protocol or modifications thereof:

PCR amplification is performed in 25 µl reactions containing: 10 mM Tris-HCl, pH 8.3 at 25°C; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; 0.5 unit Taq polymerase and 2.5 pmol of each primer together with template genomic DNA (50-100 ng) or cDNA. PCR cycling conditions comprise heating to 94°C for 45 seconds, followed by 35 cycles of 94°C for 20 seconds; annealing at X°C for 20 seconds (where X is a temperature between 40 and 70°C defined by the primer annealing temperature); 72°C for 30 seconds to several

minutes (depending on the expected length of the amplification product). The last cycle is followed by heating to 72°C for 2-3 minutes, and terminated by incubation at 4°C.

Pfam consensus: a consensus sequence derived from a large collection of protein multiple sequence alignments and profile hidden Markov models used to identify conserved protein domains (Bateman *et al.*, 2002, Nucleic Acids Res. 30: 276-80; and searchable on http://www.sanger.ac.uk/Software/Pfam/ and on NCBI at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

Protein domain prediction: sequences are analysed by BLAST (www.ncbi.nlm.nih.gov/BLAST/) and PredictProtein (www.embl-heidelberg.de/predictprotein/predictprotein.html). Signal peptides are predicted by SignalP v. 1.1 (www.cbs.dtu.dk/services/signalP/) and transmembrane regions are predicted by TMHMM v. 2.0 (www.cbs.dtu.dk/services/TMHMM/)

Polymorphism: refers to "DNA polymorphism" due to nucleotide sequence differences between aligned regions of two nucleic acid sequences.

Polynucleotide molecule: or "polynucleotide", or "polynucleotide sequence" or "nucleic acid sequence" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known analogs of natural nucleotides, which have similar binding properties as the reference nucleic acid.

Promoter: is an array of nucleic acid control sequences that direct transcription of an operably linked nucleic acid. As used herein, a "plant promoter" is a promoter that functions in plants. Promoters include necessary nucleic acid sequences near the start site of transcription, e.g. a TATA box element, and optionally includes distal enhancer or repressor elements, which can be located several 1000bp upstream of the transcription start site. A tissue specific promoter is one which specifically regulates expressed in a particular cell type or tissue e.g. roots. A "constitutive" promoter is one that is active under most environmental and developmental conditions throughout the plant.

RACE/5'RACE/3'RACE: Rapid Amplification of cDNA Ends is a PCR-based technique for the amplification of 5' or 3' regions of selected cDNA sequences which facilitates the generation of full-length cDNAs from mRNA. The technique is performed using the following standard protocol or modifications thereof: mRNA is reverse transcribed with RNase H^- Reverse Transcriptase essentially according to the protocol of Matz *et al*, (1999) *Nucleic Acids Research* 27: 1558-60 and amplified by PCR essentially according to the protocol of Kellogg *et al* (1994) *Biotechniques* 16(6): 1134-7.

Real-time PCR: a PCR-based technique for the quantitative analysis of mRNA species in an RNA preparation. The formation of amplified DNA products during PCR cycling is monitored in real-time, using a specific fluorescent DNA binding-dye and measuring fluorescence emission.

Sexual cross: refers to the pollination of one plant by another, leading to the fusion of gametes and the production of seed.

SMART consensus: represents the consensus sequence of a particular protein domain predicted by the Simple Modular Architecture Research Tool database (Schultz, J. *et al.* (1998)- *PNAS* 26;95(11):5857-64)

Southern hybridisation: Filters carrying nucleic acids (DNA or RNA) are prehybridized for 1-2 hours at 65°C with agitation in a buffer containing 7 % SDS, 0.26 M Na_2HPO_4, 5 % dextrane-suphate, 1 % BSA and 10µg/ml denatured salmon sperm DNA. Then the denatured, radioactively labelled DNA probe is added to the buffer and hybridization is carried out over night at 65°C with agitation. For low stringency, washing is carried out at 65°C with a buffer containing 2XSSC, 0.1 % SDS for 20 minutes. For medium stringency, washing is continued at 65°C with a buffer containing 1XSSC, 0.1 % SDS for 2x 20 minutes and for high stringency filters are washed a further 2x 20 minutes at 65°C in a buffer containing 0.3XSSC, 0.1 % SDS.

Probe labelling by random priming is performed essentially according to Feinberg and Vogelstein (1983) *Anal. Biochem.* 132(1), 6-13 and Feinberg and Vogelstein (1984) Addendum. *Anal. Biochem.* 137(1), 266-267

Substantially identical: refers to two nucleic acid or polypeptide sequences that have at least 60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity when aligned for maximum correspondence over a comparison window as measured using one of the sequence comparison algorithms given herein, or by manual alignment and visual inspection. This definition also refers to the complement of the test sequence with respect to its substantial identity to a reference sequence. A comparison window refers to any one of the number of contiguous positions in a sequence (being anything from between about 20 to about 600, most commonly about 100 to about 150) which may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Optimal alignment can be achieved using computerized implementations of alignment algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis. USA) or BLAST analyses available on the site: (www.ncbi.nlm.nih.gov/)

TAC clones: clones from a Transformation-competent Artificial Chromosome library.

TM marker: is a microsatellite marker developed from a TAC sequence, based on sequence differences between *Lotus japonicus* Gifu and MG-20 genotypes.

Transgene: refers to a polynucleotide sequence, for example a "transgenic expression cassette", which is integrated into the genome of a plant by means other that a sexual cross, commonly referred to as transformation, to give a transgenic plant.

UTR: untranslated region of an mRNA or cDNA sequence.

Variant: refers to "variant NFR1 or NFR5 polypeptides" encoded by different *NFR* alleles.

Wild type: a plant gene, genotype, or phenotype predominating in the wild population or in the germplasm used as standard laboratory stock.

II. Nod-factor binding

The present invention provides a Nod-factor binding element comprising one or more isolated NFR polypeptides. The isolated NFR polypeptides, NFR1 and NFR2, as exemplified by SEQ ID No: 25 and SEQ ID No: 8, bind to Nod-factors in a chemically-specific manner, distinguishing between the different chemically modified forms of Nod-factors produced by different *Rhizobium* strains. The chemical specificity of Nod-factor binding by NFR1 and NFR5 polypeptides is located in their extracellular domain, which comprises LysM type motifs. The LysM protein motif, first identified in bacterial lysin and muramidase enzymes degrading cell wall peptidoglycans, is widespread among prokaryotes and eukaryotes (Pontig et al. 1999, J Mol Biol.289, 729-745; Bateman and Bycroft, 2000, J Mol Biol, 299, 1113-1119). In bacteria it is often found in proteins associated with bacterial cell walls or involved in pathogenesis and *in vivo* and *in vitro* studies of *Lactococcus lactis* autolysin demonstrate that the three LysM domains of this protein bind peptidoglycan (Steen et al, 2003, J Biol. Chem. April issue). Since both A- and B-type peptidoglycans, differing in amino acid composition as well as cross-linking were bound, it was concluded that autolysin LysM domains binds the N-acetyl-glucosamine-N-acetyl-mureine backbone polymer LysM domains are frequently found together with amidase, protease or chitinase motifs and two confirmed chitinases carry LysM domains. One is the sex pheromone and wound-induced polypeptide from the alga *Volvox carteri* that binds and degrades chitin *in vitro* (Amon et al.1998,Plant Cell 10,781-9).The other is α-toxin from *Kluyveromyces lactis*, that docs onto a yeast cell wall chitin receptor (Butler,.et al.(1991) Eur J Biochem 199, 483-8). Structure-based alignment of representative LysM domain sequences have shown a pronounced variability among their primary sequence, except the amino acids directly involved in maintaining the secondary structure.

The NFR polypeptides are transmembrane proteins, able to transduce signals perceived by the extracellular NFR domain across the membrane to the intracellular NFR domain comprising kinase motifs, which serves to

couple signal perception to the common symbiotic signalling pathway leading to nodule development and nitrogen fixation.

The methods employed for the practise and understanding of the invention, which are described below, involve standard recombinant DNA technology that are well-known and commonly employed in the art and available from Sambrook *et al.*, 1989, *Molecular Cloning: A laboratory manual.*

III. Isolation of nucleic acid molecules comprising *NRF* genes and cDNAs encoding NFR1 and NFR5 polypeptides and their orthologues.

The isolation of genes and cDNAs encoding NFR1 and NFR5 polypeptides, comprising an amino acid sequence substantially similar to SEQ ID No: 25 or SEQ ID No: 8 respectively, may be accomplished by a number of techniques. For instance, a BLAST search of a genomic or cDNA sequence bank of a desired legume plant species (e.g. soybean, pea or *Medicago truncatula*) can identify test sequences similar to the *NFR1* or *NFR5* reference sequence, based on the smallest sum probability score (P(N)). The (P(N)) score (the probability of the match between the test and reference sequence occurring by chance) for a "similar sequence" will be less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001. This approach is exemplified by the *Medicago truncatula* sequence (Ac126779) included in Figure 3. Oligonucleotide primers, together with PCR, can be used to amplify regions of the test sequence from genomic or cDNA of the selected plant species, and a test sequence which is similar to the full-length *NFR1* and *NFR5* gene sequences can be assembled. In the case that an appropriate gene bank is not available for the selected plant species, oligonucleotide primers, based on *NFR1* and *NFR5* gene sequences, can be used to PCR amplify similar sequences from genomic or cDNA prepared from the selected plant. Alternatively, nucleic acid probes based on *NFR1* and *NFR5* gene sequences can be hybridised to genomic or cDNA libraries prepared from the selected plant species using standard conditions, in order to identify clones comprising sequences similar to *NFR1*

or *NFR5* genes. A nucleic acid sequence in a library, which hybridises to a *NFR1* or *NFR5* gene-specific probe under conditions which include at least one wash in 2xSSC at a temperature of at least about 65°C for 20 minutes, is potentially a similar sequence to a *NFR1* or *NFR5* gene. A test sequence comprising a full-length cDNA sequence similar to *NFR1* or *NFR5* cDNAs having SEQ ID No: 22 and 22 or SEQ ID no: 6 respectively, can be generated by 5' RACE cDNA synthesis, as described herein.

The nucleic acid sequence of each test sequence, derived from a selected plant species, is determined in order to identify nucleic acid molecules which are substantially identical to *NFR1* or *NFR5* genes having SEQ ID No: 23 or SEQ ID No: 7Y respectively, or nucleic acid molecules that encode proteins whose amino acid sequence is substantially identical to NFR1 or NFR5, having SEQ ID No: 25 or SEQ ID No. 8, respectively.

IV. Transgenic plants expressing NFR1 and/or NFR5 polypeptides

The polynucleotide molecules of the invention can be used to express a Nod-factor binding element in non-nodulating plants and thereby confer the ability to bind Nod-factors and establish a *Rhizobium*/plant interaction leading to nodule development. An expression cassette comprising a nucleic acid sequence encoding a NFR polypeptide, substantially identical to SEQ ID No: 25 or SEQ ID No: 8, and operably linked to its own promoter or a heterologous promoter and 3' terminator can be transformed into a selected host plant using a number of known methods for plant transformation. By way of example, the expression cassette can be cloned between the T-DNA borders of a binary vector, and transferredinto an *Agrobacterium tumerfaciens* host, and used to infect and transform a host plant. The expression cassette is commonly integrated into the host plant in parallel with a selectable marker gene giving resistance to an herbicide or antibiotic, in order to select transformed plant tissue. Stable integration of the expression cassette into the host plant genome is mediated by the virulence functions of the *Agrobacterium* host. Binary vectors and *Agrobacterium tumefaciens*-

based methods for the stable integration of expression cassettes into all major cereal plants are known, as described for example for rice (Hiei *et al.*, 1994, *The Plant J.* 6: 271-282) and maize (Yuji *et al.*, 1996, *Nature Biotechnology,* 14: 745-750). Alternative transformation methods, based on direct transfer can also be employed to stably integrate expression cassettes into the genome of a host plant, as described by Miki *et al.*, 1993, "Procedure for introducing foreign DNA into plants", In: *Methods in Plant Molecular Biology and Biotechnology,* Glick and Thompson, eds., CRC Press, Inc., Boca Raton, pp 67-88). Promoters to be used in the expression cassette of the invention include constitutive promoters, as for example the 35S CaMV promoter ((Acc V00141 and J02048) or in the case or a cereal host plant the Ubi1 gene promoter (Christensen *et al.*, 1992, *Plant Mol Biol* 18: 675-689). In a preferred embodiment, a root specific promoter is used in the expression cassette, for example the maize *zmGRP3* promoter (Goodemeir et al. 1998, Plant Mol Biol, 36, 799.802) or the epidermis expressed maize promoter described by Ponce et al. 2000, Planta, 211, 23-33. Terminators that may be used in the expression construct can for instance be the NOS terminator (Acc NC_003065).

Host plants transformed with an expression cassette encoding one NFR polypeptide, for example NFR1, or its orthologue, can be crossed with a second host plant transformed with an expression cassette encoding a second NFR polypeptide, for example NFR, or its orthologue. Progeny expressing both said NFR polypeptides can then be selected and used in the invention. Alternatively, host plants can be transformed with a vector comprising two expression cassettes encoding both said NFR polypeptides.

V. *NFR* genes encoding NFR polypeptide having specific Nod-factor binding properti s.

Nucleic acid molecules comprising *NFR1* or *NFR5* genes encoding NFR polypeptides having specific Nod-factor binding properties can be identified

by a number of functional assays described in the "Examples" given herein. In a preferred embodiment, said nucleic acid sequences are expressed transgenically in a host plant employing the expression cassettes described above. Expression of *NFR1* or *NFR5* genes or their homologous/orthologues in plant roots allows the specific Nod-factor binding properties of the expressed NFR protein to be fully tested. Assays suitable for establishing specific Nod-factor binding include the detection of: a morphological root hair response (e.g. root hair deformation, root hair curling); a physiological response (e.g. root hair membrane depolarisation, ion fluxes, pH changes and calcium oscillations); a symbiotic signalling response (e.g. downstream activation of symbiotic nodulin gene expression) following root infection with *Rhizobium* bacteria or isolated Nod-factors; the ability to develop root nodule primordia, infection pockets or root nodules, where the response is strain dependent or dependent on the chemical modification of Nod-factor structure.

VI. Marker assisted breeding for *NFR* alleles.

A method for marker assisted breeding of *NFR* alleles, encoding variant NFR polypeptides, is described herein, with examples from *Lotus NFR* alleles. In summary, variant NFR1 or NFR5 polypeptides, comprising an amino acid sequence substantially similar to SEQ ID No: 25 or SEQ ID No: 8 respectively, are identified in a nodulating legume species, and the *Rhizobium* strain specificity of said variant NRF1 or NFR5 polypeptide is determined, according to measurable morphological or physiological parameters described herein. Subsequently, DNA polymorphisms at loci genetically linked to, or within, the gene locus encoding said variant NFR1 or NFR5 polypeptide, are identified on the basis of the nucleic acid sequence of the loci or its neighbouring DNA region. Molecular markers based on said DNA polymorphisms, are used for the identification and selection of plants carrying *NFR* alleles encoding said variant NFR1 or NFR5 polypeptides. Use

of this method provides a powerful tool for the breeding of legumes with enhanced nodulation frequency.

III. Examples

Example 1.

Cloning of Nod-factor Binding Element Genes

Genetic studies in the legume plants *Lotus japonicus* (*Lj*) and pea (*Ps*) have generated collections of symbiotic mutants, which have been screened for mutants blocked in the early steps of symbiosis (Geurts and Bisseling, 2002 *supra*; Kistner and Parniske 2002 *Trends in Plant Science* 7: 511-518). Characteristic for a group of the selected mutants is their inability to respond to Nod-factors, with the absence of root hair deformation and curling, cortical cell division to form the cortical primordium, and induction of the early nodulin genes which contribute to nodule development and function. Nod-factor induced calcium oscillations were also found to be absent in some of these mutants, indicating that they are blocked in an early step in Nod-factor signalling. Among this latter group, are a few mutants, including members of the *Pssym10* complementation group and *LjNFR1* and *LjNFR5* (previously called *Ljsym1 and 5)*, which failed to respond to Nod-factors but retain their ability to establish mycorrhizal associations. Genetic mapping indicates that pea *SYM10* and *Lotus NFR5* loci in the pea and *Lotus* could be orthologs. Mutants falling within this group provided a useful starting point in the search for genes encoding potential candidate proteins involved in Nod-factor binding and perception.

A. Isolation, cloning and characterisation of *NFR5* genes and gene products.

1. Map based cloning of *Lj NFR5*

The symbiotic mutants of *Lotus japonicus nfr5-1*, *nfr5*-2 and *nfr5*-3 (also known as *sym5*), (previously isolated by Schauser *et al* 1998 *Mo. Gen Genet*, 259: 414-423; Szczglowski *et al* 1998, *Mol Plant-Microbe Interact*, 11: 684-697) were utilised. To determine the root nodulation phenotype under symbiotic conditions, seeds were surface sterilised in 2% hyperchlorite, washed and inoculated with a two day old culture of *M. loti* NZP2235. Plants were cultivated in the nitrogen-free B&D nutrients and scored after 6-7 weeks (Broughton and Dilworth, Biochem J, 1971, 125, 1075-1080; Handberg and Stougaard, Plant J. 1992, 2,487-496). Under non-symbiotic conditions, plants were cultivated in Hornum nutrients (Handberg and Stougaard, Plant J. 1992, 2,487-496).

Mapping populations were established in order to localise the *nfr5* locus on the *Lotus japonicus* genome. Both intra- and interspecific F2 mapping populations were created by crossing a *Lotus japonicus* "Gifu" *nfr5-1* mutant to wild type *Lotus japonicus* ecotype "MG20" and to wild type *Lotus filicaulis*. MG-20 seeds are obtainable from Sachiko ISOBE, National Agricultural Research Center for Hokkaido Region, Hitsujigaoka, Toyohira, Sapporo Hokkaido 062-8555, JAPAN and *L. filicaulis* from Jens Stougaard, Department of Molecular Biology, University of Aarhus, Gustav Wieds Vej 10, DK-8000 Aarhus C. F2 plants homozygous for the *nfr5-1* mutant allele were identified after screening for the non-nodulation mutant phenotype. 240 homozygous F2 mutant plants were analysed in the *L. filicaulis* mapping population and 368 homozygous F2 mutant plants in the "MG20" mapping population.

Positional cloning of the *nfr5* locus was performed by AFLP and Bulked Segregant Analysis of the mapping populations using the *Eco*RI/*Mse*I restriction enzyme combination (Vos et al, 1995, Nucleic Acids Res.23, 4407-4414; Sandal et al 2002, Genetics, 161, 1673-1683). Initially, *nfr5* was mapped to the lower arm of chromosome 2 between AFLP markers E33M40-22F and E32M54-12F in the *L. filicaulis* based mapping population, as

shown in Figure 1a . The E32M54-12F marker was cloned and used to isolate BAC clones BAC8H12 and BAC67I22 and TAC clone LjT18J10, as shown in Figure 1b. The ends of this contig were used to isolate adjacent BAC and TAC clones namely BAC58K7 and LjT01C03 at one end and TAC LjB06D23 on the other end. The outer end of LjB06D23 was used to isolate TAC clone LjT13I23. The outer end of LjB06D23 was used to isolate TAC clone LjT13I23 (TM0522). Various markers from this contig were mapped on the mapping populations from *nfr5-1* crossed to *L. filicaulis* and to *L. japonicus* MG-20. In the *L. filicaulis* mapping population one recombinant plant was found with the outer end of the TAC clone TM0522, whereas no recombinant plants were found with a marker from the middle of this TAC clone. In the *L. japonicus* MG-20 mapping population, 4 recombinant plants out of 368 plants were found with the marker TM0323, thereby delimiting *nfr5* to a region of 150 kb. This region was sequenced and found to contain 13 ORFs, of which two encoded putative proteins sharing sequence homology to receptor kinases. Sequencing of these two specific ORFs in genomic DNA derived from *nfr5-1* showed that one of the ORF sequences contained a 27 nucleotide deletion. Furthermore sequencing of this ORF in genomic DNA from *nfr5-2* and *nfr5-3* showed the insertion of a retrotransposon and a point mutation leading to a premature stop codon, respectively, as shown in Figure 1d. The localisation of the *nfr5* locus from physical and genetic mapping data, combined with the identification of mutations in three independent *nfr5* mutant alleles, provides unequivocal evidence that mutations in the *NFR5* ORF lead to a loss of Nod-factor perception.

2. Cloning the *Lj NFR5* cDNA

A full-length cDNA corresponding to the *NFR5* gene was isolated using a combination of 5'and 3' RACE. RNA was extracted from *Lotus japonicus* roots, grown in the absence of nitrate or rhizobia, and reverse transcribed to make a full-length cDNA pool for the performance of 5'-RACE according to the standard protocol. The cDNA was amplified using the 5' oligonucleotide

5'CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT 3' (SEQ ID No:1) and the reverse primer 5'GCTAGTTAAAAATGTAATAGTAACCACGC3' (SEQ ID No: 2), and a RACE-product of approximately 2 kb was cloned into a topoisomerase activated plasmid vector (Shuman, 1994, *J Biol Chem* 269: 32678-32684). 3'-RACE was performed on the same 5'-RACE cDNA pool, using a 5' gene-specific primer 5' AAAGCAGCATTCATCTTCTGG 3' (SEQ ID No: 3) and an oligo-dT primer 5'GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV 3' (SEQ ID No: 4), where the first 5 PCR cycles were carried out at an annealing temperature of 42° C and the following 30 cycles at higher annealing temperature of 58°C. The products of this PCR reaction were used as template for a second PCR reaction with a gene-specific primer positioned further 3' having the sequence 5' GCAAGGGAAGGTAATTCAG 3' (SEQ ID No: 5) and the above oligo dT-primer, using standard PCR amplification conditions (annealing at 54° C; extension 72° C for 30 s) and the products cloned into a topoisomerase activated plasmid vector (Shuman, 1994, *supra*). Nucleotide sequencing of 18 5'RACE clones and three 3' RACE clones allowed the full-length sequence of the *NFR5* cDNA to be determined (SEQ ID No: 6). The NFR5 cDNA was 2283 nucleotides in length, with an open reading frame of 1785 nucleotides, preceded by a 5' UTR leader sequence of 140 nucleotides and a 3'UTR region of 358 nucleotides. Alignment of the *NFR5* cDNA sequence with the *NFR5* gene sequence (SEQ ID No: 7), shown schematically in Figure 1d, confirmed that the gene is devoid of introns.

3. Primary sequence and structural domains of LjNFR5 and mutant alleles.

The primary sequence and domain structure of NFR5, encoded by *NFR5*, are consistent with a transmembrane Nod-factor binding protein, required for Nod-factor perception in rhizobial-legume symbiosis. The *NFR5* gene encodes an NFR5 protein of 596 amino acids having the sequence given in

Figure 2b (SEQ ID No: 8) and a predicted molecular mass of 65.3 kD. The protein domain structure predicted for NFR5 and shown in Figure 2a,b, defines a signal peptide, comprising a hydrophobic stretch of 26 amino acids, followed by an extracellular domain with three LysM-type motifs, a transmembrane domain and an intracellular kinase domain. The LysM-type motifs found in *Lotus* NFR5, SYM10, *Medicago truncatula* (*M.t*, Ac126779), and by homology in a rice gene (Ac103891), show homology to the single LysM motif present in an algal (*Volvox carteria*) chitinase (Amon *et al*, 1998, *Plant Cell* 10: 781-789) and to the Pfam consensus, as illustrated in the amino acid sequence alignment of this domain given in Figure 2c. The NFR5 kinase domain has motifs characteristic of functional serine/threonine kinases (Schenk and Snaar-Jagalska, 1999, *Biochim Biophys Acta* 1449: 1-24; Huse and Kuriyan, 2002, *Cell* 109: 275-282), with the exception that motif VII lacks an aspartic acid residue conserved in kinases, and motif VIII, comprising the activation loop, is either divergent or absent.

Analysis of the *nfr5* mutant genes reveals that the point mutation in *nfr5-3* and the retrotransposon insertion in *nfr5-2* will express truncated polypeptides of 54 amino acids, lacking the LysM motifs and entire kinase domain; or of 233 amino acids, lacking the kinase motifs X and XI, respectively. The 27 nucleotide deletion in the *nfr5-1* mutant removes 9 amino acids from kinase motif V.

4. Cloning and characterisation of the pea *SYM10* gene and cDNA and *sym10* mutants.

Wild type pea cv's (Alaska, Finale, Frisson, Sparkle) and the symbiotic mutants (N15; P5; P56) were obtained from the pea germ-plasm collection at JIC Norwich-UK, while the symbiotic mutant, RisFixG, was obtained from Kjeld Engvild, Risø National Laboratory, 8000 Roskilde, Denmark . The mutants, belonging to the pea *sym10* complementation group, were identified in the following genetic backgrounds: N15 type strain in a Sparkle

background (Kneen *et al*, 1994, *J Heredity 85: 129-133*), P5 in a Frisson background (Duc and Messager, 1989, *Plant Science* 60: 207-213), RisFixG in a Finale background RisFixG (Engvild,1987, *Theoretical Applied Genetics* 74: 711-713; Borisov *et al.*, 2000, *Czech Journal Genetics and Plant Breeding* 36: 106-110); P56 in a Frisson background (Sagan et al.1994, *Plant Science* 100: 59-70).

A fragment of the pea *SYM10* gene was cloned by PCR amplification of cv Finale genomic DNA using a standard PCR cycling program and the forward primer 5′-ATGTCTGCCTTCTTTCTTCCTTC-3′, (SEQ ID No: 9) and the reverse primer 5′-CCACACATAAGTAATMAGATACT-3′, (SEQ ID No: 10). The sequence of these oligonucleotide primers was based on nucleotide sequence stretches conserved in *L. japonicus NFR5* and the partial sequence of an *NFR5* homologue identified in a *M. truncatula* root EST collection (BE204912). The identity of the amplified 551 base pair *SYM10* product was confirmed by sequencing, and then used as a probe to isolate and sequence a pea cv Alaska *SYM10* genomic clone (SEQ ID No:11) from a cv. Alaska genomic library (obtained from H. Franssen, Department of Molecular Biology, Agricultural University, 6703 HA Wageningen, The Netherlands) and a full-length pea cv. Finale *SYM10* cDNA clone (SEQ ID No: 12) from a cv. Finale cDNA library (obtained from H. Franssen, *supra*), which were then sequenced. The sequence of the *SYM10* gene in cv. Frisson (SEQ ID No:13) and in cv. Sparkle (SEQ ID No: 14) were determined by a PCR amplification and sequencing of the amplified gene fragment. The nucleotide sequence of the corresponding mutants P5, P56, and RisFixG were also determined by a PCR amplification and sequencing of the amplified gene fragment.

Nucleotide sequence comparison of the *SYM10* gene in the *Pssym10* mutant lines (P5, RisFix6 and P56) with the wild type parent lines revealed, in each case, sequence mutations, which could be correlated with the mutant

phenotype. The 3 independent *sym10* mutant lines identified 3 mutant alleles of the *SYM10* gene, all carrying nonsense mutations, and the N15 type strain was deleted for SYM10 (Table 1, Figure 4c). Southern hybridization with probes covering either the extracellular domain of *SYM10* or the 3'UTR on *Eco*RI digested DNA from N15 and the parent variety Sparkle, shows that the *SYM10* gene is absent from the N15 mutant line.

5. Primary sequence and structural domains of PsSYM10 and mutant alleles.

The PsSYM10 protein of pea, encoded by *PsSYM10,* is a homologue of the NFR5 transmembrane Nod-factor binding protein of *Lotus*, required for Nod-factor perception in rhizobial-legume symbiosis. The pea cv Alaska *SYM10* gene encodes a SYM10 protein (SEQ ID No: 15) of 594 amino acid residues, with a predicted molecular mass of 66 kD, which shares 73% amino acid identity with the NFR5 protein from *Lotus*. In common with the NFR5 protein, the SYM10 protein has an N-terminal signal peptide, an extracellular region with three LysM motifs, followed by a transmembrane domain, and then an intracellular domain comprising kinase motifs (Figure 2 and 3).

The *sym10* genes in the symbiotic pea mutants P5, RisFix6 and P56, each having premature stop codons, encode truncated SYM10 proteins of 199, 387 and 404 amino acids, respectively, which lack part of, or the entire, kinase domain (Table 1).

6. The NFR5 protein family is unique to nodulating plants

Comparative analysis defines LjNFR5 and PsSYM10 as members of a novel family of transmembrane Nod-factor binding proteins. A BLAST search of plant gene sequences suggests that genes encoding related, but presently uncharacterised, proteins may be present in the legume *Medicago truncatula* (Ac126779), while more distantly related, predicted proteins may be found in rice (Ac103891) and *Arabidopsis* (At2g33580), with a sequence identity to NFR5 of 61%, 39%, and 28%, respectively. The high level of sequence

conservation in *M. truncatula* (Ac126779) makes this protein and the gene encoding the protein substantially identical to NFR5. In common with the NFR5 and SYM10, the kinase domains of these proteins also lack the conserved aspartic acid residue of motif VII, and the activation loop in motif VIII is highly diverged or absent, as shown in Figure 2d, with the exception of the *Arabidopsis* protein. Only distantly related proteins are therefore found outside the legume family. In conclusion, the NFR5 protein family appears to be restricted to nodulating legumes, and its absence from other plant families may be a key limiting factor in the establishment of rhizobial-root interactions in the members of the families.

7. Tissue specific expression of the *LjNFR5* and *PsSYM10* genes

The expression pattern of the *NFR5* and *SYM10* genes in *Lotus* and pea is consistent with the role of their gene products as transmembrane Nod-factor binding proteins in the perception of rhizobial Nod-factors at the root surface and later during tissue invasion.

The expression of the *NFR5* and *SYM10* genes in various isolated organs of *Lotus* and pea plants, was investigated by determining the steady state *NFR5* and *SYM10* mRNA levels using Real-time PCR and/or Northern blot analysis. Total RNA was isolated from root, leaf, flower, pod and nodule tissues of uninoculated or inoculated *Lotus* "Gifu" or pea plants using a high salt extraction buffer followed by purification through a CsCl cushion. For Northern analysis, according to standard protocols, 20 μg total RNA was size-fractionated on 1.2% agarose gel, transferred to a Hybond membrane, hybridised overnight with an *NFR5* or *SYM10* specific probe covering the extracellular domain and washed at high stringency. Hybridization to the constitutively expressed ubiquitin *UBI* gene was used as control for RNA loading and quality of the RNA.

For the quantitative real-time RT-PCR, total RNA was extracted using the CsCl method and the mRNA was purified by biomagnetic affinity separation

(Jakobsen, K.S. *et al* (1990) *Nucleic Acids Research* 18(12): 3669). The RNA preparations were analysed for contaminating DNA by quantitative PCR and when necessary, the RNA was treated with DNaseI. The DNaseI enzyme was then removed by phenol:chloroform extraction and the RNA was precipitated and re-suspended in 20 µl RNase free H_2O. First strand cDNA was prepared using Expand reverse transcriptase and the quantitative real-time PCR was performed on a standard PCR LightCycler instrument. The efficiency-corrected relative transcript concentration was determined and normalized to a calibrator sample, using *Lotus japonicus* ATP synthase gene as a reference (Gerard C.J. *et al*, 2000 *Mol. Diagnosis* 5: 39-45).

The level of *NFR5* mRNA, determined by Northern blot analysis and quantitative RT-PCR, was 60 to 120 fold higher in the root tissue of *Lotus* plants in comparison to other plant tissues (leaves, stems, flowers, pods, and nodules), as shown in Figure 4a. Northern hybridisation show highest expression of *NFR5* in *Lotus* root tissue and a barely detectable expression in nodules. Northern blot analysis detected *SYM10* mRNA in the roots of pea, and a higher level in nodules, but no mRNA was detected in leaves, as shown in Figure 4c.

B. Isolation, cloning and characterisation of *NFR1* genes and gene products.

1. Map based cloning of *Lj NFR1*

The *NFR1* gene was isolated using a positional cloning approach. On the genetic map of *Lotus* the *NFR1* locus is located on the short arm of chromosome I, approximately 22 cM from the top, within a 7.6 cM interval, as shown in Figure 5a. Several TM markers and PCR markers, derived from DNA polymorphism in the genome sequences of the *L. japonicus* mapping parents, were found to be closely linked to *NFR1* locus and were used to narrow down the region. A physical map of the region, comprising a contig of

assembled BAC and TAC clones, is shown in figure 5b. Fine mapping in an F2 population, established from a *Lotus japonicus nfr-1* mutant to wild type *L. japonicus* ecotype 'Miyakojima MG-20' cross, and genotyping of 1603 mutant plants, identified two markers (56K22, 56L2-2) delimiting the *NFR1* locus within a region of 250 kb. BAC and TAC libraries, available from Satoshi Tabata, Kazusa DNA Research Institute, Kisarazu, Chiba 292-0812 Japan; another BAC library from Jens Stougaard, Department of Molecular Biology, University of Aarhus, Gustav Wieds Vej 10, DK-8000 Aarhus C, were screened using the closest flanking markers (56L2-1,10M24-1, 36D15) as probes, and the *NFR1* locus was localised to 36 kb within the region. The ORFs detected within the region coded for a UFD1-like protein, a hypothetical protein and a candidate NFR1 protein showing homology to receptor kinases, (Figure 5b).

The region in the genomes of *nfr1-1, nfr1-2* mutants, corresponding to the candidate *NFR1* gene was amplified as three fragments by PCR under standard conditions and sequenced. The fragment of 1827 bp amplified using PCR forward primer 5'TGC ATT TGC ATG GAG AAC C3', (SEQ ID No: 16) and reverse primer 5' TTT GCT GTG ACA TTA TCA GC3', (SEQ ID No: 17) contains single nucleotide substitutions leading to translational stop codons in both the mutant alleles *nfr1-1,* with a CAA to TAA substitution, and the *nfr1-2,* with a GAA to TAA substitution. The physical and genetic mapping of the *nfr1* locus, combined with the identification of mutations in two independent *nfr1* mutant alleles, provides unequivocal evidence that the sequenced *NFR1* gene is required for Nod-factor perception and subsequent signal transduction.

2. Cloning the *Lj NFR1* cDNAs

Two alternatively spliced *Lj NFR1* cDNAs were identified using a combination of cDNA library screening and 5' RACE on root RNA from *Lotus japonicus.* A *Lotus* root cDNA library (Poulsen *et al.*, 2002, *MPMI* 15:376-379) was screened with an *NFR1* gene probe generated by PCR amplification of the

nucleotides between 9689 to 10055 of the genomic sequence, using the primer pair: 5' TTGCAGATTGCACAACTAGG3' (SEQ ID No: 18) and 5'ACTTAGAATCTGCAACTTTGC 3' (SEQ ID No: 19). Total RNA extracted from *Lotus* roots, was amplified by 5' RACE, according to the standard protocol, using the gene specific reverse primer 5'ACTTAGAATCTGCAACTTTGC 3' (SEQ ID No 20). Based on the sequence of isolated *NFR1* cDNAs and 5' RACE products, the *NFR1* gene produces two mRNA species, of 2187 (SEQ ID No: 21) and 2193 nucleotides (SEQ ID No: 22), with a 5' leader sequence of 114 nucleotides, and a 3' untranslated region is 207 nucleotides (Figure 5c). Alignment of genomic and cDNA sequences defined 12 exons in *NFR1* and a gene structure spanning 10235 bp (SEQ ID No: 23). The sequenced region includes 4057bp from the stop codon of the previous gene up to the transcription start point of NFR1 + 6009 bp of NFR1 + 187 bp of 3'genomic. Alternative splice donor sites at the 3'of exon IV account for the two alternative *NFR1* mRNA species.

3. Primary sequence and structural domains of LjNFR1 and mutant alleles.

The primary sequence and domain structure of NFR1, encoded by *LjNFR1*, are consistent with a transmembrane Nod-factor binding protein, required for Nod-factor perception in *Rhizobium*-legume symbiosis. The alternatively spliced *NFR1* cDNAs encode NFR1 proteins of 621 (SEQ ID No: 24) and 623 amino acids (SEQ ID No: 25), with a predicted molecular mass of 68.09 kd and 68.23 kd, respectively. The protein has an amino-terminal signal peptide, followed by an extracellular domain having two LysM-type motifs, a transmembrane domain, and an intracellular carboxy-terminal domain comprising serine/threonine kinases motifs

In *nfr1-1*, a stop codon in kinase domain VIII encodes truncated polypeptides of 490 and 492 amino acids, and in *nfr1-2* a stop codon between domain IX and XI encodes truncated polypeptides of 526 and 528 amino acids, as indicated in Figure 6a.

In Figure 6b the M1 LysM motif of NFR1 is aligned with the LysM motifs from *Arabidopsis thaliana* and the SMART consensus and M2 LysM of NFR1 with the *Volvox carteri* chitinase (Acc No: T08150), the closest related *Arabidopsis thaliana* receptor kinase (Acc No: NP_566689) , the rice (Acc No: BAB89226) and the consensus SMART LysM motif.

4. The LjNFR1 protein family is not found in non-nodulating plants

Comparative analysis defines LjNFR1 as a member of a second novel family of transmembrane Nod-factor binding proteins. Although proteins having both receptor-like kinase domains and LysM motifs are predicted from plant genome sequences, their homology to NFR1 is low and their putative function unknown. *Arabidopsis* has five predicted receptor-like kinases with LysM motifs in the extracellular domain, and one of them (At3g21630) is 54% identical to NFR1 at the protein level. Rice has 2 genes in the same class, and one (BAB89226) encodes a protein with 32 % identity to NFR1. This suggests that the NFR1 protein is essential for Nod-factor perception and its absence from non-nodulating plants may be a key limiting factor in the establishment of rhizobial-root interactions in these plants. Although NFR1 shares the same domain structure to NFR5 their primary sequence homology is low (Figure 11).

5. Expression of the *LjNFR1, NFR5 and SymRK* symbiotic genes is root specific and independently regulated.

The *NFR1* dependent root hair curling, in the susceptible zone located just behind the root tip, is correlated with root specific *NFR1* gene expression. Steady-state *NFR1* mRNA levels were measured in different plant organs using quantitative real-time PCR and Northern blot analysis as described above in section A.7. *NFR1* mRNA was only expressed in root tissue, and remained below detectable levels in leaves, flowers, pods and nodules, as shown in Figure 7a. Upon inoculation with *M. loti,* the expression of *NFR1* in wild type plants is relatively stable for at least 12 days after inoculation

(Figure 7b). Real-time PCR experiments revealed no difference between the levels of the two *NFR1* transcripts detected in the root RNA, suggesting that the alternative splicing of exon 4 is not differentially regulated.

NFR1, *NFR5* and *SymRK* gene expression in roots, before and following *Rhizobium* inoculation, was determined by real-time PCR in wild type and *nfr1, nfr5 and symrk* mutant genotypes. The expression of *NFR1, NFR5* and *SymRK* genes in un-inoculated and inoculated roots was not significantly influenced by the symbiotic mutant genotype (Figure 7b, c, d) indicating that transcriptional regulation of these genes is mutually independent.

Example 2.

Functional properties of the Nod-factor binding element and its component NFR proteins

The functional and regulatory properties of the Nod-factor binding element and its component NFR proteins provide valuable tools for monitoring the functional expression and specific activity of the NFR proteins. Nod-factor perception by the Nod-factor binding element triggers the rhizobial-host interaction, which includes depolarisation of the plasma membrane, ion fluxes, alkalization of the external root hair space of the invasion zone, calcium oscillations and cytoplasmic alkalization in epidermal cells, root hair morphological changes, infection thread formation and the initiation of the nodule primordia. These physiological events are accompanied and coordinated by the induction of specific plant symbiotic genes, called nodulins. For example, the *NIN* gene encodes a putative transcriptional regulator facilitating infection thread formation and inception of the nodule primordia and limits the region of root cell-rhizobial interaction competence to a narrow invasion zone (Geurts and Bisseling, 2002, *supra*). Since *nin* mutants develop normal mycorrhiza, the *NIN gene* lies in the rhizobia-specific branch of the symbiotic signalling pathway, downstream of the common pathway. Ion fluxes, pH changes, root hair deformation and nodule formation

are all absent in *NFR1* and *NFR5* mutant plants, and hence the functional activity of these genes must be required for all downstream physiological responses. Several physiological and molecular markers that are diagnostic of *NFR* expression are provided below.

1. Morphological marker of *NFR1* and *NFR5* gene expression

When wild type *Lotus japonicus* plants are inoculated with *Mesorhizobium loti*, the earliest visible evidence of infection is root hair deformation and root hair curling, which occurs 24 hours after inoculation, as shown in Figure 8a. However, mutant plants carrying the *nfr1-1* (Figure 8c), *nfr1-2*, *nfr5-1*, *nfr* 5-2 or *nfr*5-3 alleles (as in Figure 8c), all failed to produce root hair curling or deformation, infection threads or nodule primordia in response to infection by *Mesorhizobium loti* with all three strains tested (NZP2235, R7A and TONO). Lipochitin-oligosaccharides purified from *M. loti*, R7A strain, which induce root hair deformation and branching in wild type plants (Figure 8b), also failed to induce any deformation of root hairs of the *nfr1-1* and *nfr5-1* mutants (Figure 8d), evidencing the key role of the *NFR1* and *NFR5* genes in Nod-factor perception.

Mutations in genes expressing the downstream components of the symbiosis signalling pathway, namely *symRK and nin* have clearly distinguishable phenotypes. After infection with *Mesorhizobium loti*, the root hairs of *symRK* plants swell into balloon structures (Figure 8e), while the *nin* mutants produce an excessive root hair response (Figure 8g). The response of double mutants carrying *nfr1-1/symRK-3* mutant alleles or *nfr1-1/ nin* alleles to *Mesorhizobium loti* infection (Figure 8f,h) are similar to that of *nfr1-1* mutants, demonstrating that the *nfr1-1* mutation is dominant to *symRK* and *nin* mutations, and hence determines an earlier step in the symbiotic signalling pathway.

2. Physiological marker of *NFR1* and *NFR5* gene expression

When the root hairs of wild type *Lotus* plants are exposed to *M. loti* Nod-factor, the plasma membrane is depolarised and an alkalisation occurs in the root hair space of the invasion zone, (Figure 9a). The extracellular pH was monitored continuously in a flow-through regime using a pH-selective microelectrode, placed within the root hair space. Membrane potential was measured simultaneously with pH, and the calculated values are based on at least three equivalent experiments, each. Mutants carrying *nfr1* and *nfr5* alleles do not respond normally to Nod-factor stimulation. Two *nfr5* alleles abolish the response to Nod-factors (Figure 9b), while the *nfr1-1* allele causes a diminished and slower alkalisation, and the *nfr1-2* allele causes the acidification of the extracellular root hair space (Figure 9c). Both the *NFR1* and *NFR5* genes are thus essential for mounting the earliest detectable cellular and electrophysiological responses to Nod-factor, which can be used to monitor their functional activity.

The early physiological response of the *symRK-3 and symRK-1* mutant plants to *Mesorhizobium loti* Nod-factor is similar to the wild type (Figure 9d) and clearly distinguishable from the response of both the *nfr*1 and *nfr5* mutants.

The response of the double mutant, carrying *nfr1-2/symRK-3* mutant alleles, to Nod-factor (Figure 9e) is similar to that of *nfr1-2* mutants, further supporting that the *nfr1-2* mutation is dominant to *symRK-3* and determines an earlier step in the symbiotic signalling pathway.

3. *NFR1* and *NFR5* mediated Nod-factor perception lies upstream of *NIN* and *ENOD* and is required for their expression.

The symbiotic expression of the nodulin genes, *Lotus japonicus ENOD2* (Niwa, S. *et al.*, 2001 *MPMI* 14:848-56) and *NIN*, in roots following rhizobial inoculation, provides a marker for *NFR* gene expression. The steady-state levels of *NIN* and *ENOD2* mRNA were measured in roots before and

following rhizobial inoculation by quantitative real-time PCR, using the primer pairs:
5'AATGCTCTTGATCAGGCTG3' (SEQ ID No: 26) and
5'AGGAGCCCAAGTGAGTGCTA3' (SEQ ID No: 27) for amplification of *NIN* mRNA reverse transcripts; and the primer pairs:
5'CAG GAA AAA CCA CCA CCT GT3' (SEQ ID No:28) and
5'ATGGAGGCGAATACACTGGTG3' (SEQ ID No: 29) for amplification of *ENOD2* mRNA reverse transcripts. The identity of the amplified sequences was confirmed by sequencing.

Five hours after inoculation, induction of *NIN* gene expression was detected in the wild type plants, while induction of *ENOD2* occurs after 12 days as shown in Figure 10a and b. In the *nfr1* and *nfr5* mutants, activation of *NIN* and *ENOD2* was not detected, demonstrating that functional *NFR1* and *NFR5* genes can be monitored by the activation of these early nodulin genes.

Lotus plants transformed with a *NIN* gene promoter region fused to a GUS reporter gene provide a further tool to monitor *NFR* gene function. Expression of the *NIN*-GUS reporter can be induced in root hairs and epidermal cells of the root invasion zone following rhizobial inoculation in transformed wild-type plants. In contrast expression of the *NIN*-GUS reporter in an *nfr1* mutant was not detected following rhizobial inoculation. Likewise, *NIN*-GUS expression was induced in the invasion zone of wildtype plants after Nod-factor application, while in a *nfr1* mutant background no expression was detected The requirement for *NFR1* function was confirmed in *nfr1-1*, *nin* double mutants by the absence of root hair curling and excessive root hair curling (Fig 8).

The *LjCBP1* gene, T-DNA tagged with a promoter-less GUS in the T90 line, is rapidly activated after *M. loti* inoculation as seen for *NIN*-GUS, thus providing an independent and sensitive reporter of early nodulin gene expression (Webb et al, 2000, Molecular Plant-Microbe Interact. 13,606,-616). Parallel experiments comparing expression of the *LjCBP1* promoter GUS fusion in wt and *nfr1* mutant background confirm the requirement for a

functional *NFR1* for activation of the early response to bacteria and Nod-factor.

Example 3.

Transgenic expression of NFR polypeptides and complementation of the *nfr* mutants

The *NFR* genes, encoding the NFR1 and NFR5 protein components of the Nod-factor binding element, can each be stabily integrated, as a transgene, into the genome of a plant, such as a non-nodulating plant or a mutant non-nodulating plant, by transformation. Expression of this transgene, directed by an operably linked promoter, can be detected by expression of the respective NFR protein in the transformed plant and functional complementation of a non-nodulating mutant plant.

A wildtype *NFR5* transgene expression cassette of 3,5 kb, comprising a 1175 bp promotor region, the *NFR5* gene and a 441 bp 3' UTR was cloned in a vector (pIV10), and the vector was recombined into the T-DNA of *Agrobacterium rhizogenes* strain AR12 and AR1193 by triparental mating. The *NFR5* expression cassette in pIV10 was subsequently transformed into non-nodulating *Lotus nfr5-1* and *nfr5*-2 mutants via *Agrobacterium rhizogenes*-mediated transformation according to the standard protocol (Stougaard 1995, Methods in Molecular Biology volume 49, Plant Gene Transfer and Expression Protocols, p 49-63) In parallel, control transgenic *Lotus nfr5-1* and *nfr5*-2 mutants plants were generated, which were transformed with an empty vector, lacking the *NFR5* expression cassette. The nodulation phenotype of the transgenic hairy root tissue of the transformed mutant *Lotus* plants was scored after inoculation with *Mesorhizobium loti* (*M. loti*) strain NZP2235. *In planta* complementation of the *nfr5-1* and *nfr5*-2 mutants by the *NFR5* transgene was accomplished, as shown in Table 2, with an efficiency of ≥58%, and the establishment of normal rhizobial-legume interactions and development of nitrogen fixing

nodules. Complementation was dependent on transformation with a vector comprising the *NFR5* expression cassette.

A transgene expression cassette, comprising the wild type *NFR1* gene comprising 3020 bp of promoter region, the *NFR1* ORF and 394 bp of 3'untranslated region, was cloned into the pIV10 vector and recombined into *Agrobacterium rhizogenes* strain AR12 and AR1193 by triparental mating. *Agrobacterium rhizogenes*-mediated transformation was used to transform the gene into non-nodulating *Lotus nfr1-1* and *nfr1-2* mutants in parallel with a control empty vector. *In planta* complementation of the *Lotus nfr1-1* and *nfr1-2* mutants by the *NFR1* transgene was accomplished, as shown in Table 3, with an efficiency of ≥60%, and the establishment of normal *Rhizobium*-legume interactions with *M. loti* strain NZP2235, and development of nitrogen fixing nodules. Complementation was dependent on transformation with a vector comprising the *NFR1* expression cassette

Example 4

Expression and characterisation of the NFR1, NFR5 and SYM10 proteins in transgenic plants

NFR1, NFR5 and SYM10 proteins are expressed and purified from transgenic plants, by exploiting easy and well described transformation procedures for *Lotus* (Stougaard 1995, *supra*) and tobacco (Draper et al.1988, Plant Genetic Transformation and Gene Expression, A Laboratory Manual, Blackwell Scientific Publications). Expression in plants is particularly advantageous, since it facilitates the correct folding of these transmembrane proteins and provides for correct post-translational modification, such as phosphorylation. The primary sequences of the expressed proteins are extended with commercially available epitope tags (Myc or FLAG), to allow their purification from plant protein extracts. DNA sequences encoding the tags are ligated into the expression cassette for each protein, in frame, either

at the 5′ or the 3′ end of the cDNA coding region. These modified coding regions are then operably linked to a promoter, and recombined into *Agrobacterium rhizogenes. Lotus* is transformed by wound-site infection and from the transgenic roots independent root cultures are established *in vitro* (Stougaard 1995, *supra*). NFR1, NFR5 and SYM10 proteins are then purified from root cultures by affinity chromatography using the epitope specific antibody and standard procedures. Alternatively the proteins are immunoprecipitated from crude extracts or from semi-purified preparations. Proteins are detected by Western blotting methods. For transformation and expression in tobacco, the epitope tagged cDNAs are cloned into an expression cassette comprising a constitutively expressed 35S promoter and a 3′UTR and subsequently inserted into binary vectors. After transfer of the binary vector into *Agrobacterium tumefaciens*, transgenic tobacco plants are obtained by the transformation regeneration procedure (Draper et al.1988, *supra*). Proteins are then extracted from crude or semi-purified extracts of tobacco leaves using affinity purification or immunoprecipitation methods. The epitope tagged purified protein preparations are used to raise mono-specific antibodies towards the NFR1, NFR5 and SYM10 proteins

Example 5

Plant breeding tools to select for enhanced nodulation frequency and efficiency.

A successful and efficient primary interaction between a rhizobial strain and its host depends on detection of a *Rhizobium* strain's unique Nod-factor (LCO) profile by the plant host. The Nod-factor binding element and its component NFR proteins, each with their extracellular LysM motifs, play a key role in controlling this interaction. *NFR* alleles, encoding variant NFR proteins are shown to be correlated with the efficiency and frequency of

nodulation with a given rhizobial strain. Molecular breeding tools to detect and distinguish different plant *NFR* alleles, and assays to assess the nodulation efficiency and frequency of each allele, provides an effective method to breed for nodulation efficiency and frequency.

Methods useful for breeding for nodulation efficiency and frequency are given below, and the application of these techniques is illustrated for the *NFR* alleles of *Lotus* spp. Using the *Rhizobium leguminosarum* bv *viceae* 5560 DZL strain (Bras et al, 2000, Molecular Plant-Microbe Interact. 13, 475-479) it is documented that the host range of this strain within the *Lotus* spp depends on the *NFR1* and *NFR5* alleles present in the *Lotus* host. When inoculated onto wild type plants *Rhizobium leguminosarum* bv *viceae* 5560 DZL form root nodules on *Lotus japonicus* GIFU but the strain is unable to form root nodules on *Lotus filicaulis*. Transgenic *L. filicaulis* transformed with the *Lotus japonicus* GIFU *NFR1* and *NFR5* alleles do however form root nodules when inoculated with the *Rhizobium leguminosarum* bv *viceae* 5560 DZL strain proving the NFR1/NFR5 allele dependent Nod-factor recognition.

1. Determining the Nod-factor specificity and sensitivity of *NFR alleles*.

Root hair curling and root hair deformation in the susceptible invasion zone is a sensitive *in vivo* assay for monitoring the legume plants ability to recognise a *Rhizobium* strain or the Nod-factor synthesized by a *Rhizobium* strain. The assay is performed on seedlings and established as follows. Seeds of wild type, transgenic and mutant *Lotus* spp are sterilised and germinated for 3 days. Seedlings are grown on 1/4 B&D medium (Handberg and Stougaard, 1992 *supra*), between two layers of sterile wet filter paper for 3 days more. Afterwards, they are transferred into smaller petri dishes containing 1/4 B&D medium supplemented with 12.7nM AVG [(S)-trans-2-amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride] (Bras C. et al, 2000 , *MPMI* 13: 475-479). On transfer, the seedlings are inoculated with either 20 µl of 1:100

dilution of a 2 days old *M.loti* strain NZP2235 culture, or with *M.loti* strain R7A Nod-factor coated sand, or with sterile water as a control, and a layer of wet dialysis membrane is used to cover the whole root. A minimum of 30 seedlings are microscopically analysed for specific deformations of the root hairs. The assay determines the threshold sensitivity of each *L. japonicus* , for the Nod-factor (LCO) of a given *Rhizobium* strain and the frequency of root hair curling and/or deformation.

In an alternative procedure, seeds of *Lotus japonicus* are surface sterilised and germinated for 4 days on 1% agar plates containing half-strength nitrogen-free medium (Imaizumi-Anraku *et al.*, 1997, *Plant Cell Physiol.* 38: 871-881), at 26°C, under a 16h light and 8h dark regime. Straight roots, of <1cm in length, on germlings from each cultivar are then selected and transplanted on Fåhraeus slides, in a nitrogen-free medium and grown for a further 2 days. LCOs, prepared by *n*-butanol extraction and HPLC separation from a given *Rhizobium* strain (Niwa *et al.* 2001, *MPMI* 14: 848-856), are applied to the straight roots in each cultivar, at a final concentration range of between 10^{-7} and 10^{-9} M. After 12 to 24h culture, the roots are stained with 0.1% toluene blue and the number of root hairs showing curling is counted. The assay determines the threshold sensitivity of each *Lotus* spp., carrying a given *NFR* allele, for the Nod-factor (LCO) of a given *Rhizobium* strain and the frequency of root hair curling.

2. Determining the frequency and efficiency of nodulation of *NFR* alleles.

The efficiency of a legume plants ability to form root nodules after inoculation with a *Rhizobium* strain is determined in small scale controlled nodulation tests. *Lotus* seeds are surface sterilised in 2 % hyperchlorite and cultivated under aseptic conditions in nitrogen free 1/4 concentrated B&D medium. After 3 days of germination, seedlings are inoculated with a 2 days old

culture of *M. loti* NZP2235 or TONO or R7A or with the *R. leguminosarum* bv *viceae* 5560DZL strain. In principle a set of plants is only inoculated with one stain. For controlled competition experiments where legume-*Rhizobium* recognition is determined in a mixed *Rhizobium* population, a set of plants can be inoculated with more than one *Rhizobium* strain or with an extract from a particular soil. Two growth regimes are used: either petri dishes with solidified agar or Magenta jars with a solid support of burnt clay and vermiculite. The number of root nodules developed after a chosen time period is then counted, and the weight of the nodules developed can be determined. The efficiency of the root nodules in terms of nitrogen fixation can be determined in several ways, for example as the weight of the plants or directly as the amount of N15 nitrogen incorporated in the plant molecules.

In an alternative procedure, *Lotus* seeds are surface sterilised and vernalised at 4°C for 2 days on agar plates and germinated overnight at 28°C. The seedlings are inoculated with *Mesorhizobium loti* strain NZP2235, TONO or R7A LCOs (as described above) and grown in petri dishes on Jensen agar medium at 20°C in 8h dark, 16h light regime. The number of nodules present on the plant roots of each cultivar is determined at 3 days intervals over a period of 25 days, providing a measure of the rate of nodulation and the abundance of nodules per plant.

3. Determining nodule occupancy in relation to *NFR* allele

In agriculture the NFR Nod-factor binding element recognises *Rhizobium* bacteria under adverse soil conditions. The final measure of a particular strain's or commercial *Rhizobium* inoculum's ability to compete with the endogenous *Rhizobium* soil population for invasion of a legume crop with particular NFR alleles, is root nodule occupancy. The proportion of nodules formed after invasion by a particular strain and the fraction of the particular *Rhizobium* strain inside individual root nodules is determined by surface

sterilising the root nodule surface in hyperchlorite, followed by crushing of the nodule into a crude extract and counting the colony forming *Rhizobium* units after dilution of the extract and plating on medium allowing *Rhizobium* growth (Vincent., JM. 1970, A manual for the practical study of root nodule bacteria. IBP handbook no. 15 Oxford Blackwell Scientific Publications, López-García *et al*, 2001, *J Bacteriol*, 183,7241-7252).

4. *NFR1 and NFR5* are determinants of host range in *Lotus-Rhizobium* interactions.

Wild type *Lotus japonicus* Gifu is nodulated by both *Rhizobium leguminosarum bv. viciae* 5560 DZL (*R. leg* 5560DZL) and *Mesorhizobium loti* NZP2235 (*M.loti* NZP2235), while wild type *Lotus filicaulis* is only nodulated by *M.loti* NZP2235. Transgenic *Lotus filicaulis* plants expressing the *NFR1* and *NFR5* alleles of *Lotus japonicus* Gifu, are nodulated by *R. leg* 5560DZL, clearly demonstrating that the *NFR* alleles are the primary determinants of host range.

Lotus filicaulis was transformed with vectors comprising *NFR1* and *NFR5* wild type genes and their cognate promoters from *Lotus japonicus* Gifu or with empty vectors. The *Lotus filicaulis* transformants carrying *NFR1* and *NFR5* are nodulated by *R. leg* 5560DZL, albeit at reduced efficiency/frequency (9.6%) compared to *Lotus japonicus* Gifu (100%), as shown in Table 4. Mixing of NFR subunits from *Lotus japonicus* and *Lotus filicaulis* in the Nod-factor binding element is likely to contribute to the reduced efficiency observed. These data demonstrate that rhizobial strain recognition specificity is determined by the *NFR1* and *NFR5* alleles and that breeding for specific *NFR* alleles present in the germplasm or in wild relatives can be used to select optimal legume-*Rhizobium* partners.

More detailed investigations show that the rhizobial strain recognition specificity of the *NFR5* and *NFR1* alleles is determined by the extracellular domain of the NFR5 and NFR1 proteins. Mutant *Lotus japonicus nfr5* was transformed with a wild type hybrid *NFR5* gene "FinG5", encoding the extracellular domain from *L. filicaulis* NFR5 fused to the kinase domain from *L. japonicus* Gifu NFR5 (Figure 12). The hybrid gene was operably linked to the wild type *NFR5* promoter. Control transformants, comprising wild type *L. japonicus* Gifu, L. *filicaulis* and the *Lotus japonicus nfr5* mutant, transformed with an empty vector, are generated in parallel. The transformed plants are infected either with *M.loti* NZP2235 or with *R. leg*5560 DZL and the formation of nodules monitored, as shown in Table 5. The FinG5 hybrid gene complements the *nfr5* mutation, and 88% of the transformants are nodulated by *M.loti* NZP2235 showing that the hybrid gene is functionally expressed. However, the *nfr5* mutants expressing the FinG5 hybrid gene are very poorly nodulated by *R.leg* 5560 DZL, only 3 %, (corresponding to one plant) even after prolonged infection (40 days). This demonstrates that strain specificity of the Nod-factor binding element is determined by the extracellular domain of its component NFR proteins.

In parallel, the *Lotus japonicus nfr1* mutant was transformed with a wild type hybrid *NFR1* gene "FinG1", encoding the extracellular domain from *L. filicaulis* NFR1 fused to the kinase domain from *L. japonicus* Gifu NFR1 (Figure 12). The hybrid gene was operably linked to the wild type *NFR1* promoter. The transformed plant were infected either with *M.loti* NZP2235 or with *R. leg* 5560 DZL and the formation of nodules was monitored, as shown in Table 6.

The FinG1 hybrid gene complements the *nfr1-1* mutation, and 100 % of the transformants were nodulated by *M.loti* NZP2235. However *nfr1-1* mutants expressing the FinG1 hybrid gene were less efficiently nodulated (30-40%) by *R. leg* 5560 DZL. Furthermore, their nodulation by *R. leg* 5560 DZL was much delayed compared to their nodulation by *M. loti* NZP2235. Thus the

Lotus / *R. leg* 5560 DZL interaction is less efficient and delayed when the transgenic host plant expresses a hybrid NFR1 comprising the extracellular domain of *Lotus filicaulis* NFR1 with the kinase domain of *Lotus japonicus* Gifu NFR1. These data indicate that the specific recognition of *R.leg 5560* DZL by its *Lotus* host is at least partly specified by the extracellular domain of NFR1 (Gifu) and that this is an allele specific recognition. However, the *NFR5* allele appears to be more important for specific recognition than *NFR1*.

5. *NFR5* alleles and their molecular markers

The NFR5 Nod-factor binding proteins encoded by the *NFR5* alleles of *Lotus japonicus* ecotype GIFU (gene sequence: SEQ ID No: 7; protein sequence: SEQ ID No: 24 & 25), and *Lotus filicaulis* (gene sequence SEQ ID No: 30; protein sequence SEQ ID No: 31) have been compared, and found to show diversity in their primary structure. Using the sequence information available for the *Lotus NFR5* gene together with the pea SYM10 gene (Table 8), the alleles from different ecotypes or varieties of *Lotus*, pea and other legumes can now be identified, and used directly in breeding programs. Molecular markers based on DNA polymorphism are used to detect the alleles in breeding populations. Similar use can be taken of the *NFR1* sequences. Molecular DNA markers, based on the *NFR5* allele sequence differences of *Lotus* and pea, are highlighted in Tables 8 and 9 as examples of how DNA polymorphism can be used directly to detect the presence of an advantageous allele in a breeding population.

Breeding for an advantageous allele can also be carried out using molecular markers, that are genetically linked to the allele of interest, but located outside the gene-allele itself. Breeding of new *Lotus japonicus* lines containing a desired *NFR5* allele can, for example, be facilitated by the use of DNA polymorphisms, (simple sequence repeats (microsatelittes) or single nucleotide polymorphism (SNP) which are found at loci, genetically linked to

NFR5. Microsatelittes and SNPs at the *NFR5* locus are identified by transferring markers from the general map, by identification of AFLP markers, or, by scanning the nucleotide sequence of the BAC and TAC clones spanning the *NFR5* locus, for DNA polymorphic sequences located in close proximity of the *NFR5* gene. Table 7 lists the markers closely linked to *NFR5* and the sequence differences used to design the microsatelitte or SNP markers. This principle of marker assisted breeding, using genetically linked markers, can be applied to all plants. Microsatellite markers which generate PCR products with a high degree of polymorphism, are particularly useful for distinguishing closely related individuals, and hence to distinguish different *NFR5 of NFR1* alleles in a breeding program.

Table 1

Summary of *Lotus nfr5* and pea *sym10* mutant alleles

Allele	Mutation	*Lotus* Spp
sym5-1	EYAENGSLA 380-388 deletion	Lj
sym5-2	retrotransposon integration after Q233	Lj
sym5-3	CAG→TAG, Q55→stop	Lj
RisFixG	TGG→TGA, W_{388}→stop	Ps
P5	TGG→TGA, W_{405}→stop	Ps
P56	CAA→TAA, Q_{200}→stop	Ps
N15	*Sym10* gene deleted	Ps

TABLE 2

Complementation of *Lotus japonicus nfr5* mutants with the wildtype *NFR5* transgene

Lotus genotype	Transgene	No. of plants	Infected With	No. of plants with nodules*	Total No. of nodules
nfr5-1	*NFR5*	31	*M.loti* NZP2235	18	nd
nfr5-1	Empty vector	20	*M.loti* NZP2235	0	nd
nfr5-2	*NFR5*	5	*M.loti* NZP2235	1	nd
nfr5-2	Empty vector	5	*M.loti* NZP2235	0	nd

* Nodules only detected on transformed roots

TABLE 3

Transformation of *Lotus japonicus nfr1* mutants with the wildtype *NFR1* transgene

Lotus genotype	Transgene	No. of plants	Infected With	No. plants with nodules	Total No. of nodules	Average No. nodules/ plant
nfr1-1	*NFR1*	103	*M.loti* NZP2235	62*	310	5
nfr1-1	Empty vector	30	*M.loti* NZP2235	0	0	0
nfr1-2	*NFR1*	20	*M.loti* NZP2235	13*	97	7.5
nfr1-2	empty vector	7	*M.loti* NZP2235	0	0	0

* Nodules only detected on transformed roots

Table 4

***Lotus filicaulis* transformed with wildtype *NFR1* and *NFR5* genes from *Lotus japonicus* Gifu**

Lotus genotype	Transgene	No. of plants	Infected with	No. plants with nodules	Total No. of nodules	Average No. nodules/ plant
Lotus filicaulis	*NFR1+ NFR5*	104	*R.leg* 5560 DZL	10*	25	2.5
Lotus filicaulis	Empty vector	65	*R.leg* 5560 DZL	0	0	0
Lotus japonicus Gifu	Empty vector	10	*R.leg* 5560 DZL	10**	>150	>15

* Nodules only detected on transformed roots

** Nodules on normal and transformed roots

Table 5

***L. japonicus nfr5* mutant transformed with a hybrid *NFR5* gene "FinG5" encoding the extracellular domain of *L.filicaulis* NFR5 fused to the kinase domain from *L. japonicus* Gifu NFR5.**

Lotus genotype	Transgene	No. of plants	Infected with	No. of plants with nodules	Total No. of nodules	Average No. nodules/ plant
nfr5	FinG5	31	*M.loti* NZP2235	28*	~180	6.4
nfr5	Empty vector	12	*M.loti* NZP2235	0	0	
nfr5	FinG5	34	*R.leg 5560* DZL	1*	4	4 1 PLANT ONLY
nfr5	empty vector	10	*R.leg 5560* DZL	0	0	
Lotus japonicus Gifu	empty vector	10	*R.leg 5560* DZL	10**	>150	>15
Lotus filicaulis	empty vector	29	*R.leg 5560* DZL	0	0	

* Nodules only detected on transformed roots

** Nodules on normal and transformed roots

Table 6

***L. japonicus nfr1* mutant transformed with a hybrid *NFR1* gene "FinG1" encoding the extracellular domain of *L.filicaulis* NFR1 fused to the kinase domain from *L. japonicus* Gifu NFR1.**

Lotus genotype	Transgene	No. of plants	Infected with	No. of plants with nodules	Total No. of nodules	Average No. nodules/ plant
nfr1-1	FinG1	8	*M.loti* NZP2235	8*	59	7.3
nfr1-1	Empty vector	6	*M.loti* NZP2235	0	0	0
nfr1-1	FinG1	13	*R. leg* 5560DZL	5*#	15	3
nfr1-1	Empty vector	9	*R. leg* 5560DZL	0	0	0
nfr1-2	FinG1	10	*R. leg* 5560DZL	3*#	12	4
nfr1-2	Empty vector	4	*R. leg* 5560DZL	0	0	0

* Nodules only detected on transformed roots

Nodules were first counted after 56 days, while *M.loti* NZP2235 nodules were detectable after ~25 days.

Table 7

Molecular markers for *NFR5* allele breeding in *Lotus*

Marker	Genetic distance from *NFR5* locus	*Lotus* Ecotype	Microsatellite sequence
TM0272	2,9cM	MG-20	18xCT
		Gifu	12xCT
TM0257	1,0cM	MG-20	10xAAG
		Gifu	7xAAG
LjT13i23Sfi		Gifu	TTTTGCTGCAGCAAGTCAGACTGTTAGAGGA
		Fili	TTTTGCTGCAACAAGTCGGACTGTTAGAGGA
TM0522	0cM	MG-20	24xAT
		Gifu	14xAT
NFR5			
E32M54-12F	0,5cM	MG-20	TTGGAAGTTCTTTTTATTAGGTTAATTTTA
		Fili	TTGGAAGTTCTTTTTA---GGTTAATTTTA
LjT01c03 Not	0,7cM	Fili	CATTCCAGAAGAAAATAAGATATAATTATG
		MG-20	CATTCCAGAAGAAAATAAGATATAATTATG
		Gifu	CATTCCAGAAG-AAATAAGATATAATTATG
TM0168	2,2cM	MG-20	19xAT
		Gifu	15xAT
TM0021	3,8cM	MG-20	16xCT
		Gifu	13xCT

Table 8

Nucleotide sequence variation between the pea *SYM10* alleles of pea cultivars Frisson and Finale*

Frisson	CTTGCATTTC TTCACAATTT CACAACAATG GCTATCTTCT TTCTTCCTTC
Finale	CTTGCATTTC TTCACAATTT CACAACAATG GCTATCTTCT TTCTTCCTTC
Frisson	TAGTTCTCAT GCCCTTTTTC TTGCACTCAT GTTTTTTGTC ACTAATATTT
Finale	TAGTTCTCAT GCCCTTTTTC TTGCACTCAT GTTTTTTGTC ACTAATATTT
Frisson	CAGCTCAACC ATTACAACTC AGTGGAACAA ACTTTTCATG CCCGGTGGAT
Finale	CAGCTCAACC ATTACAACTC AGTGGAACAA ACTTTTCATG CCCGGTGGAT
Frisson	TCACCTCCTT CATGTGAAAC CTATGTGACA TACTTTGCTC GGTCTCCAAA
Finale	TCACCTCCTT CATGTGAAAC CTATGTGACA TACTTTGCTC GGTCTCCAAA
Frisson	CTTTTTGAGC CTAACTAACA TATCAGATAT ATTTGATATG AGTCCTTTAT
Finale	CTTTTTGAGC CTAACTAACA TATCAGATAT ATTTGATATG AGTCCTTTAT
Frisson	CCATTGCAAA AGCCAGTAAC ATAGAAGATG AGGACAAGAA GCTGGTTGAA
Finale	CCATTGCAAA AGCCAGTAAC ATAGAAGATG AGGACAAGAA GCTGGTTGAA
Frisson	GGCCAAGTCT TACTCATACC TGTAACTTGT GGTTGCACTA GAAATCGCTA
Finale	GGCCAAGTCT TACTCATACC TGTAACTTGT GGTTGCACTA GAAATCGCTA
Frisson	TTTCGCGAAT TTCACGTACA CAATCAAGCT AGGTGACAAC TATTTCATAG
Finale	TTTCGCGAAT TTCACGTACA CAATCAAGCT AGGTGACAAC TATTTCATAG
Frisson	TTTCAACCAC TTCATACCAG AATCTTACAA ATTATGTGGA AATGGAAAAT
Finale	TTTCAACCAC TTCATACCAG AATCTTACAA ATTATGTGGA AATGGAAAAT
Frisson	TTCAACCCTA ATCTAAGTCC AAATCTATTG CCACCAGAAA TCAAAGTTGT
Finale	TTCAACCCTA ATCTAAGTCC AAATCTATTG CCACCAGAAA TCAAAGTTGT
Frisson	TGTCCCTTTA TTCTGCAAAT GCCCCTCGAA GAATCAGTTG AGCAAAGGAA
Finale	TGTCCCTTTA TTCTGCAAAT GCCCCTCGAA GAATCAGTTG AGCAAAGGAA
Frisson	TAAAGCATCT GATTACTTAT GTGTGGCAGG CTAATGACAA TGTTACCCGT
Finale	TAAAGCATCT GATTACTTAT GTGTGGCAGG CTAATGACAA TGTTACCCGT
Frisson	GTAAGTTCCA AGTTTGGTGC ATCACAAGTG GATATGTTTA CTGAAAACAA
Finale	GTAAGTTCCA AGTTTGGTGC ATCACAAGTG GATATGTTTA CTGAAAACAA
Frisson	TCAAAACTTC ACTGCTTCAA CCAACGTTCC GATTTTGATC CCTGTGACAA
Finale	TCAAAACTTC ACTGCTTCAA CCAATGTTCC GATTTTGATC CCTGTGACAA
Frisson	AGTTACCGGT AATTGATCAA CCATCTTCAA ATGGAAGAAA AAACAGCACT
Finale	AGTTACCGGT AATTGATCAA CCATCTTCAA ATGGAAGAAA AAACAGCACT

Frisson CAAAAACCTG CTTTTATAAT TGGTATTAGC CTAGGATGTG CTTTTTTCGT
Finale CAAAAACCTG CTTTTATAAT TGGTATTAGC CTAGGATGTG CTTTTTTCGT

Frisson TGTAGTTTTA ACACTATCAC TTGTTTATGT ATATTGTCTG AAAATGAAGA
Finale TGTAGTTTTA ACACTATCAC TTGTTTATGT ATATTGTCTG AAAATGAAGA

Frisson GATTGAATAG GAGTACTTCA TTGGCGGAGA CTGCGGATAA GTTACTTTCA
Finale GATTGAATAG GAGTACTTCA TTGGCGGAGA CTGCGGATAA GTTACTTTCA

Frisson GGTGTTTCGG GTTATGTAAG CAAGCCAACA ATGTATGAAA TGGATGCGAT
Finale GGTGTTTCGG GTTATGTAAG CAAGCCAACA ATGTATGAAA TGGATGCGAT

Frisson CATGGAAGCT ACAATGAACC TGAGTGAGAA TTGTAAGATT GGTGAATCCG
Finale CATGGAAGCT ACAATGAACC TGAGTGAGAA TTGTAAGATT GGTGAATCTG

Frisson TTTACAAGGC TAATATAGAT GGTAGAGTTT TAGCAGTGAA AAAAATCAAG
Finale TTTACAAGGC TAATATAGAT GGTAGAGTTT TAGCAGTGAA AAAAATCAAG

Frisson AAAGATGCTT CTGAGGAGCT GAAAATTTTG CAGAAGGTAA ATCATGGAAA
Finale AAAGATGCTT CTGAGGAGCT GAAAATTCTG CAGAAGGTAA ATCATGGAAA

Frisson TCTTGTGAAA CTTATGGGTG TGTCTTCCGA CAACGACGGA AACTGTTTCC
Finale TCTTGTGAAA CTTATGGGTG TGTCTTCCGA CAACGAAGGA AACTGTTTCC

Frisson TTGTTTACGA GTATGCTGAA AATGGATCAC TTGATGAGTG GTTGTTCTCA
Finale TTGTTTACGA GTATGCTGAA AATGGATCAC TTGATGAGTG GTTGTTCTCA

Frisson GAGTCGTCGA AAACTTCGAA CTCGGTGGTC TCGCTTACAT GGTCTCAGAG
Finale GAGTTGTCGA AAACTTCGAA CTCGGTGGTC TCGCTTACAT GGTCTCAGAG

Frisson AATAACAGTA GCAGTGGATG TTGCAGTTGG TTTGCAATAC ATGCATGAAC
Finale AATAACAGTA GCAGTGGATG TTGCAGTTGG TTTGCAATAC ATGCATGAAC

Frisson ATACTTACCC AAGAATAATC CACAGAGACA TCACAACAAG TAATATCCTT
Finale ATACTTACCC AAGAATAATC CACAGAGACA TCACAACAAG TAATATCCTT

Frisson CTGGATTCAA ACTTTAAGGC CAAGATAGCG AATTTTTCAA TGGCCAGAAC
Finale CTGGATTCAA ACTTTAAGGC CAAGATAGCG AATTTTTCAA TGGCCAGAAC

Frisson TTCAACAAAT TCCATGATGC CGAAAATCGA TGTTTTCGCT TTTGGGGTGG
Finale TTCAACAAAT TCCATGATGC CGAAAATCGA TGTTTTCGCT TTTGGGGTGG

Frisson TTCTGATTGA GTTGCTTACC GGCAAGAAAG CGATAACAAC GATGGAAAAT
Finale TTCTGATTGA GTTGCTTACC GGCAAGAAAG CGATAACAAC GATGGAAAAT

Frisson GGCGAGGTGG TTATTCTGTG GAAGGATTTC TGGAAGATTT TTGATCTAGA
Finale GGCGAGGTGG TTATTCTGTG GAAGGATTTC TGGAAGATTT TTGATCTAGA

Frisson AGGGAATAGA GAAGAGAGCT TAAGAAAATG GATGGATCCT AAGCTAGAGA
Finale AGGGAATAGA GAAGAGAGCT TAAGAAAATG GATGGATCCT AAGCTAGAGA

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Frisson   ATTTTTATCC TATTGATAAT GCTCTTAGTT TGGCTTCTTT GGCAGTGAAT
Finale    ATTTTTATCC TATTGATAAT GCTCTTAGTT TGGCTTCTTT GGCAGTGAAT

Frisson   TGTACTGCAG ATAAATCATT GTCAAGACCA AGCATTGCAG AAATTGTTCT
Finale    TGTACTGCAG ATAAATCATT GTCAAGACCA AGCATTGCAG AAATTGTTCT

Frisson   TTGTCTTTCT CTTCTCAATC AATCATCATC TGAACCAATG TTAGAAAGAT
Finale    TTGTCTTTCT CTTCTCAATC AATCATCATC TGAACCAATG TTAGAAAGAT

Frisson   CCTTGACATC TGGTTTAGAT GTTGAAGCTA CTCATGTTGT TACTTCTATA
Finale    CCTTGACATC TGGTTTAGAT GTTGAAGCTA CTCATGTTGT TACTTCTATA

Frisson   GTAGCTCGTT GATATTCATT CAAGTGAAGG TAACACTCAA TCAATGCTTC
Finale    GTAGCTCGTT GATATTCATT CAAGTGAAGG TAACACTAAA TCAATGCTTC

Frisson   AGTTTCTTAT ATTCAAGATG GTTACTTTGT TTAGATGATT ATTGATTACA
Finale    AGTTTCTTAT ATTCAAGATG GTTACTTTGT TTAGCTGATT ATTGATTACA

Frisson   TCTTTATGTG TGGAACTATA TGGTTATTTT AATTAAGGGA ATTGTTCTAA
Finale    TCTTTATGTG TGGAACTATA TGGTTATTTT AATTAAGGGA ATTAGTCTAA

Frisson   AATTCATTTT TCCATGTT
Finale    ATTTCATTTT TCCATGTT
```

* Nucleotide differences are shaded black and the coding region is underlined

Table 9

Protein sequence differences encoded by the pea *SYM10* alleles of pea cultivars Frisson and Finale*

Frisson	MAIFFLPSSS HALFLALMFF VTNISAQPLQ LSGTNFSCPV DSPPSCETYV
Finale	MAIFFLPSSS HALFLALMFF VTNISAQPLQ LSGTNFSCPV DSPPSCETYV
Frisson	TYFARSPNFL SLTNISDIFD MSPLSIAKAS NIEDEDKKLV EGQVLLIPVT
Finale	TYFARSPNFL SLTNISDIFD MSPLSIAKAS NIEDEDKKLV EGQVLLIPVT
Frisson	CGCTRNRYFA NFTYTIKLGD NYFIVSTTSY QNLTNYVEME NFNPNLSPNL
Finale	CGCTRNRYFA NFTYTIKLGD NYFIVSTTSY QNLTNYVEME NFNPNLSPNL
Frisson	LPPEIKVVVP LFCKCPSKNQ LSKGIKHLIT YVWQANDNVT RVSSKFGASQ
Finale	LPPEIKVVVP LFCKCPSKNQ LSKGIKHLIT YVWQANDNVT RVSSKFGASQ
Frisson	VDMFTENNQN FTASTNVPIL IPVTKLPVID QPSSNGRKNS TQKPAFIIGI
Finale	VDMFTENNQN FTASTNVPIL IPVTKLPVID QPSSNGRKNS TQKPAFIIGI
Frisson	SLGCAFFVVV LTLSLVYVYC LKMKRLNRST SLAETADKLL SGVSGYVSKP
Finale	SLGCAFFVVV LTLSLVYVYC LKMKRLNRST SLAETADKLL SGVSGYVSKP
Frisson	TMYEMDAIME ATMNLSENCK IGESVYKANI DGRVLAVKKI KKDASEELKI
Finale	TMYEMDAIME ATMNLSENCK IGESVYKANI DGRVLAVKKI KKDASEELKI
Frisson	LQKVNHGNLV KLMGVSSDND GNCFLVYEYA ENGSLDEWLF SESSKTSNSV
Finale	LQKVNHGNLV KLMGVSSDNE GNCFLVYEYA ENGSLDEWLF SELSKTSNSV
Frisson	VSLTWSQRIT VAVDVAVGLQ YMHEHTYPRI IHRDITTSNI LLDSNFKAKI
Finale	VSLTWSQRIT VAVDVAVGLQ YMHEHTYPRI IHRDITTSNI LLDSNFKAKI
Frisson	ANFSMARTST NSMMPKIDVF AFGVVLIELL TGKKAITTME NGEVVILWKD
Finale	ANFSMARTST NSMMPKIDVF AFGVVLIELL TGKKAITTME NGEVVILWKD
Frisson	FWKIFDLEGN REESLRKWMD PKLENFYPID NALSLASLAV NCTADKSLSR
Finale	FWKIFDLEGN REESLRKWMD PKLENFYPID NALSLASLAV NCTADKSLSR
Frisson	PSIAEIVLCL SLLNQSSSEP MLERSLTSGL DVEATHVVTS IVAR
Finale	PSIAEIVLCL SLLNQSSSEP MLERSLTSGL DVEATHVVTS IVAR

* Amino acid differences are highlighted in black.

Claims

1. A Nod-factor binding element comprising one or more isolated NFR polypeptides.
2. The Nod-factor binding element of claim 1, wherein the NFR polypeptide is NFR1, comprising an amino acid sequence substantially identical to SEQ ID No: 25, and having specific Nod-factor binding properties.
3. The Nod-factor binding element of claim 1, wherein the NFR polypeptide is NFR5 comprising an amino acid sequence substantially identical to SEQ ID No: 8 and having specific Nod-factor binding properties.
4. The Nod-factor binding element of claim 1, comprising the NFR polypeptides NFR1 and NFR5, comprising amino acid sequences substantially identical to SEQ ID No: 25 and SEQ ID No: 8, respectively, and having specific Nod-factor binding properties.
5. The Nod-factor binding element of claim 2 or 4, wherein said NFR1 polypeptide is encoded by an *NFR1* gene comprising a nucleic acid sequence substantially identical to SEQ ID No: 23.
6. The Nod-factor binding element of claim 3 or 4, wherein said NFR5 polypeptide is encoded by an *NFR5* gene comprising a nucleic acid sequence substantially identical to SEQ ID No: 7.
7. An isolated polynucleotide molecule comprising a nucleic acid sequence encoding the NFR1 polypeptide of claim 2 that is substantially identical to SEQ ID No: 23.
8. An isolated polynucleotide molecule comprising a nucleic acid sequence encoding the NFR5 polypeptide of claim 3 that is substantially identical to SEQ ID No: 7.
9. A method of producing a plant expressing the Nod-factor binding element of claims 2 or 3, the method comprising introducing into the plant a transgenic expression cassette comprising a nucleic acid

sequence encoding a NFR polypeptide that is substantially identical to SEQ ID No: 25 or SEQ ID No:8, and having specific Nod-factor binding properties, wherein the nucleic acid sequence is operably linked to a promoter and selecting transgenic plants and their progeny expressing said NFR polypeptide.

10. The method of claim 9, wherein the transgenic expression cassette is introduced into the plant through a sexual cross.

11. The method of claim 9, wherein said promoter is a native promoter or heterologous root specific promoter.

12. The method of claim 9, wherein said promoter is a native or heterologous constitutive promoter.

13. A transgenic plant expressing one or more NFR polypeptides produced according to the method of any one of claims 9 to 12.

14. The transgenic plant of claim 13, expressing NFR 1 and NFR5 polypeptides comprising an amino acid sequence substantially identical to SEQ ID No: 25 and SEQ ID No: 8, respectively and having specific Nod-factor binding properties.

15. The transgenic plant of claim 13, wherein the plant is a non-nodulating dicotyledenous plant.

16. The transgenic plant of claim 13, wherein the plant is a monocotyledonous cereal.

17. A method for marker assisted breeding of *NFR* alleles, encoding variant NFR polypeptides, comprising the steps of:

a. identifying variant NFR1 or NFR5 polypeptides in a nodulating legume species, comprising an amino acid sequence substantially similar to SEQ ID No: 25 or SEQ ID No: 8 respectively, having specific Nod-factor binding properties, and

b. determining the nodulation frequency of legume plants expressing said variant NRF1 or NFR5 polypeptide, and

c. identifying DNA polymorphisms at loci genetically linked to or within the allele encoding said variant NFR1 or NFR5 polypeptide, and

d. preparing molecular markers based on said DNA polymorphisms, and

e. using said molecular markers for the identification and selection of plants carrying *NFR* alleles encoding said variant NFR1 or NFR5 polypeptides.

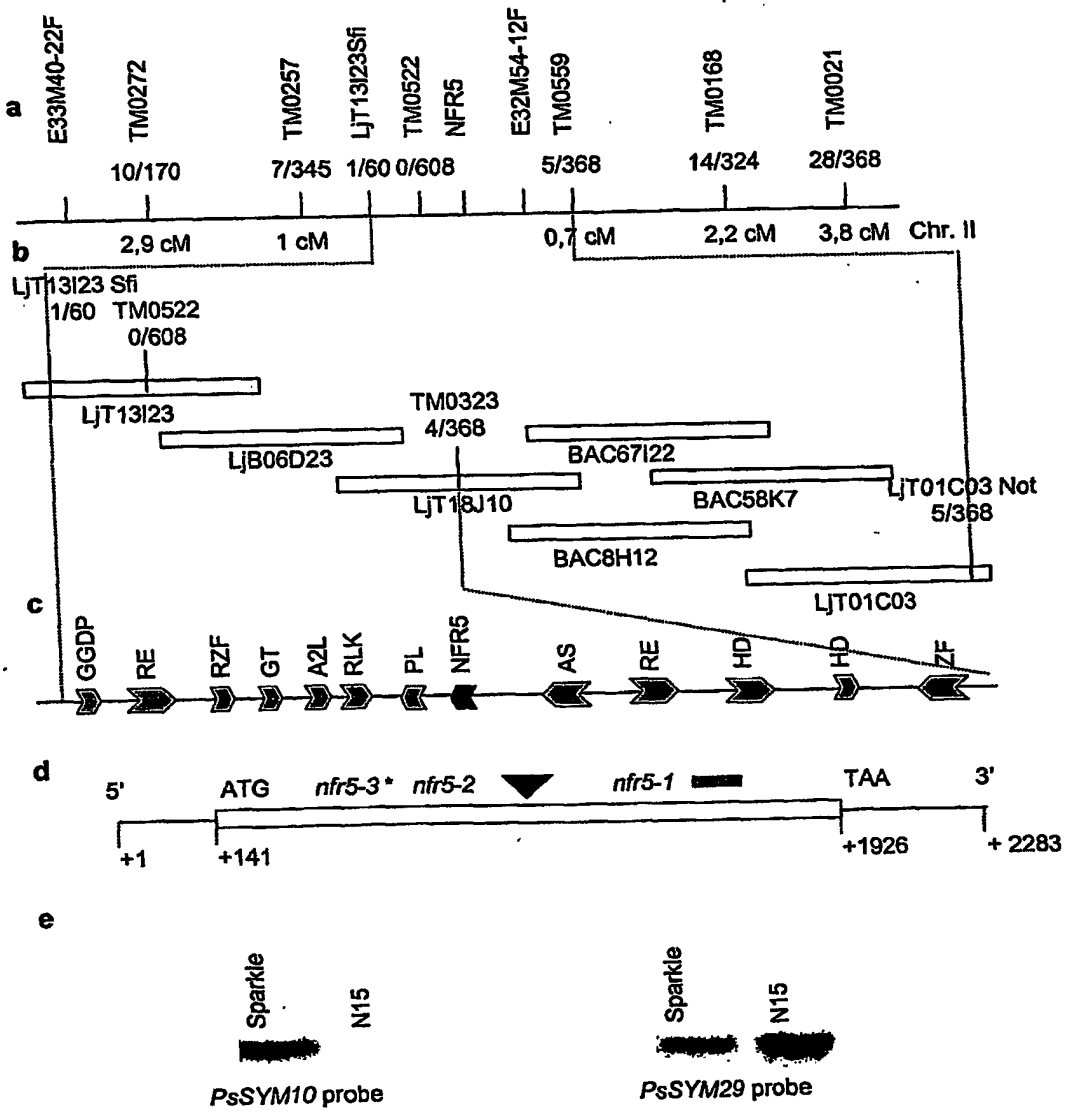
18. Plants selected according the method of claim 17, carrying *NFR* alleles encoding variant NFR1 or NFR5 polypeptides comprising an amino acid sequence substantially similar to SEQ ID No: 25 or SEQ ID No: 8, respectively, and having specific Nod-factor binding properties.

19. Use of the method of claim 16 for breeding legumes with enhanced nodulation frequency or root nodule occupancy or enhanced symbiotic nitrogen fixation ability.

Abstract

The present invention provides a Nod-factor binding element, comprising one or more NFR polypeptides encoded by *NFR* genes, that are useful for providing non-nodulating plants with Nod-factor binding properties and triggering the endosymbiotic signalling pathway leading to nodulation. Furthermore the invention is useful for breeding for improved nodulation in nodulating legumes.

Figure 1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E33M40-22F
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TM0257
LjT13I23Sfi
TM0522
NFR5
E32M54-12F
TM0559
TM0168
TM0021
10/170
7/345
1/60
0/608
5/368
14/324
28/368
2,9 cM
1 cM
0,7 cM
2,2 cM
3,8 cM
Chr. II
b
LjT13I23 Sfi
1/60
TM0522
0/608
LjT13I23
TM0323
4/368
LjB06D23
BAC67I22
LjT18J10
BAC58K7
LjT01C03 Not
5/368
BAC8H12
LjT01C03
c
GGDP
RE
RZF
GT
A2L
RLK
PL
NFR5
AS
RE
HD
HD
ZF
d
5'
ATG
nfr5-3*
nfr5-2
nfr5-1
TAA
3'
+1
+141
+1926
+ 2283
e
Sparkle
N15
PsSYM10 probe
Sparkle
N15
PsSYM29 probe

Figure 2

a

b

MAVFFLTSGSLSLFLALTLLFTNIAA **SP**

RSEKISGPDFSCPVDSPPSCETYVT **51 aa**

*

YTAQSPNLLSLTNISDIFDISPLSIARASNIDAGKDKLVPGQVLLVP **LysM1**

VTCGCAGNHSSANTS **113 aa**

YQIQLGDSYDFVATTLYENLTNWNIVQASNPGVNPYLLPERVKVVFP **LysM2**

LFCRCPSKNQLNKGIQYLIT **180 aa**

YVWKPNDNVSLVSAKFGASPADILTENRYGQDFTAATNLPILIP **LysM3**

VTQLPELTQPSSNGRKSSIHLL **246 aa**

VILGITLGCTLLTAVLTGTLVYVYC **TM**

RRKKALNRTASSAETADKLLSGVSGYVSKPNVYEIDEI **KD**

I

MEATKDFSDECKVGESVYKANIEGRVVAVKKIKEGGANEELKILQKV

II III

NHGNLVKLMGVSSGYDGNCFLVYEYAENGSLAEWLFSKSSGTPNSLT

IV V

WSQRISIAVDVAVGLQYMHEHTYPRIIHRDITTSNILLDSNFKAKIA

VIa VIb

NFAMARTSTNPMMPKIDVFAFGVLLIELLTGRKAMTTKENGEVVMLW

VII IX

KDMWEIFDIEENREERIRKWMDPNLESFYHIDNALSLASLAVNCTAD

KSLSRPSMAEIVLSLSFLTQQSSNPTLERSLTSSGLDVEDDAHIVTS

XI

ITAR **595 aa**

Figure 2

c

```
NFR5M1      52:YTAQSPNLLSLTNISDIFDISPLSIARASNIDAGKDKLVPCQVLLVP:98
SYM10M1     52:YFARSPNFLSLTNISDIFDMSPLSIAKASNIEDEDKKLVECQVLLIP:98
M.tM1       53:YRAQSPNFLSLSNISDIFNLSPLRIAKASNIEAEDKKLIPDQLLLVP:99
RiceM1      47:YRTQSPGFLDLGNISDLFGVSRALIASANKLTTEDGVLLPCQPLLVP:93

NFR5M2     114:YQIQLGDSYDFVATTLYENLTNWNIVQASNPGVNPYLLPERVKVVFP:160
SYM10M2    114:YTIKLGDNYFIVSTTSYQNLTNYVEMENFNPNLSPNLLPPEIKVVVP:160
M.tM2      115:YSIKQGDNFFILSITSYQNLTNYLEFKNFNPNLSPTLLPLDTKVSVP:161
RiceM2     109:YPIRPRDTFFGLAVIAFENLIDFVLVEELNPAAEAIRLEPWQEVVVP:155
VolvoxM2   106:YTIQPGDTFWAIAQR.RG..ITVDVIQSLNPGVNPARLQVGQVINVP:149
Pfam         1:YTVKKGDILWKIARR.YG..ISVSELKSLN.GLSSDNLYVGQKLKIP:43

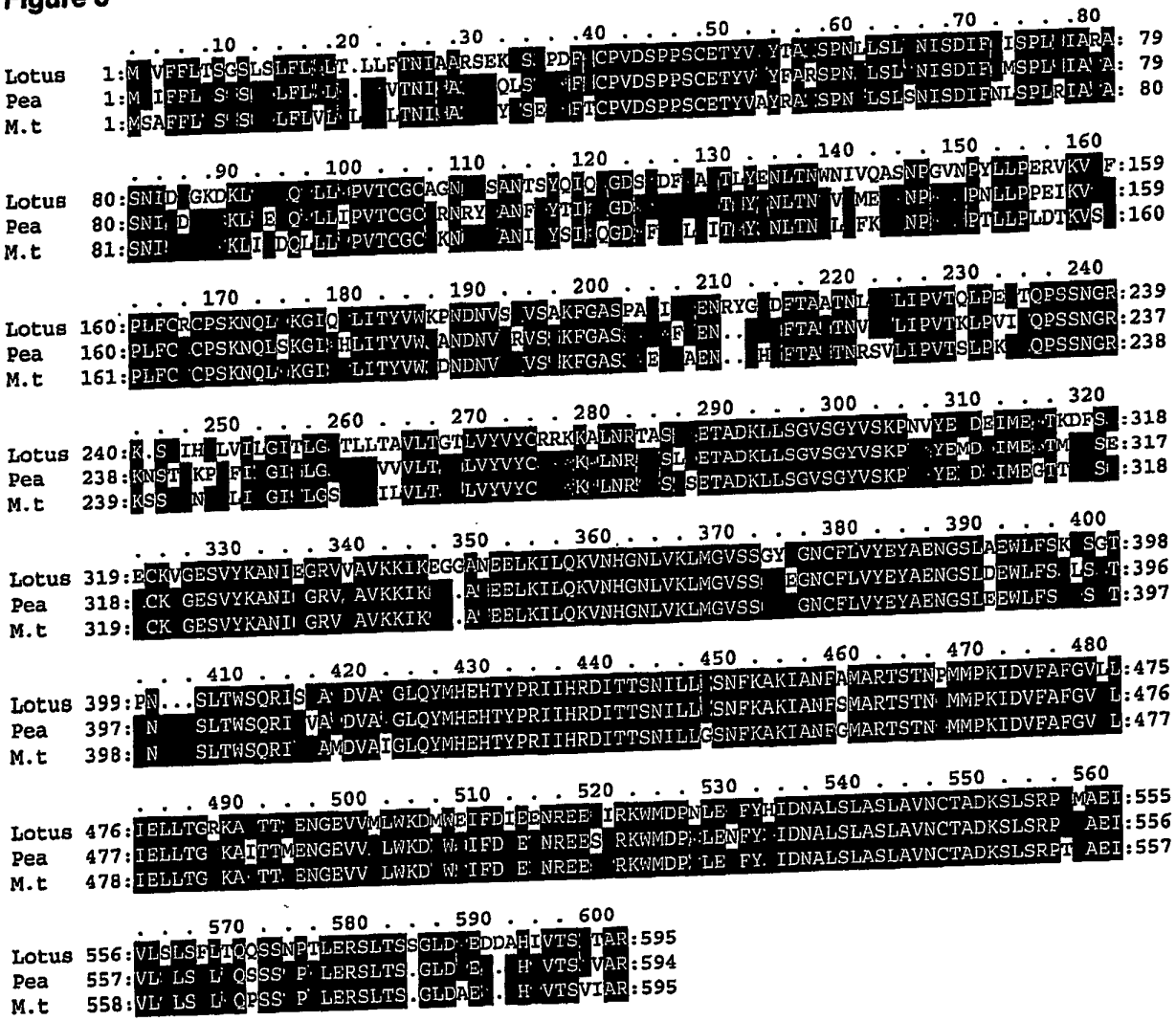
NFR5M3     181:YVWKPNDNVSLVSAKFGASPADILIENRYGQDFTAATNLPILIP:224
SYM10M3    181:YVWQANDNVTRVSSKFGASQVDMFTEN..NQNFTASTNVPILIP:222
M.tM3      182:YVWQDNDNVTLVSSKFGASQVEMLAEN..NHNGTASTNRSVLIP:223
RiceM3     176:YVWQEGDDVSVVSALMNASAANIAASNGVAGNSTFATGQPVLIP:219
```

d

```
                 VII                                VIII              IX
Cons          ...DFG.............................APE...........D.W..G

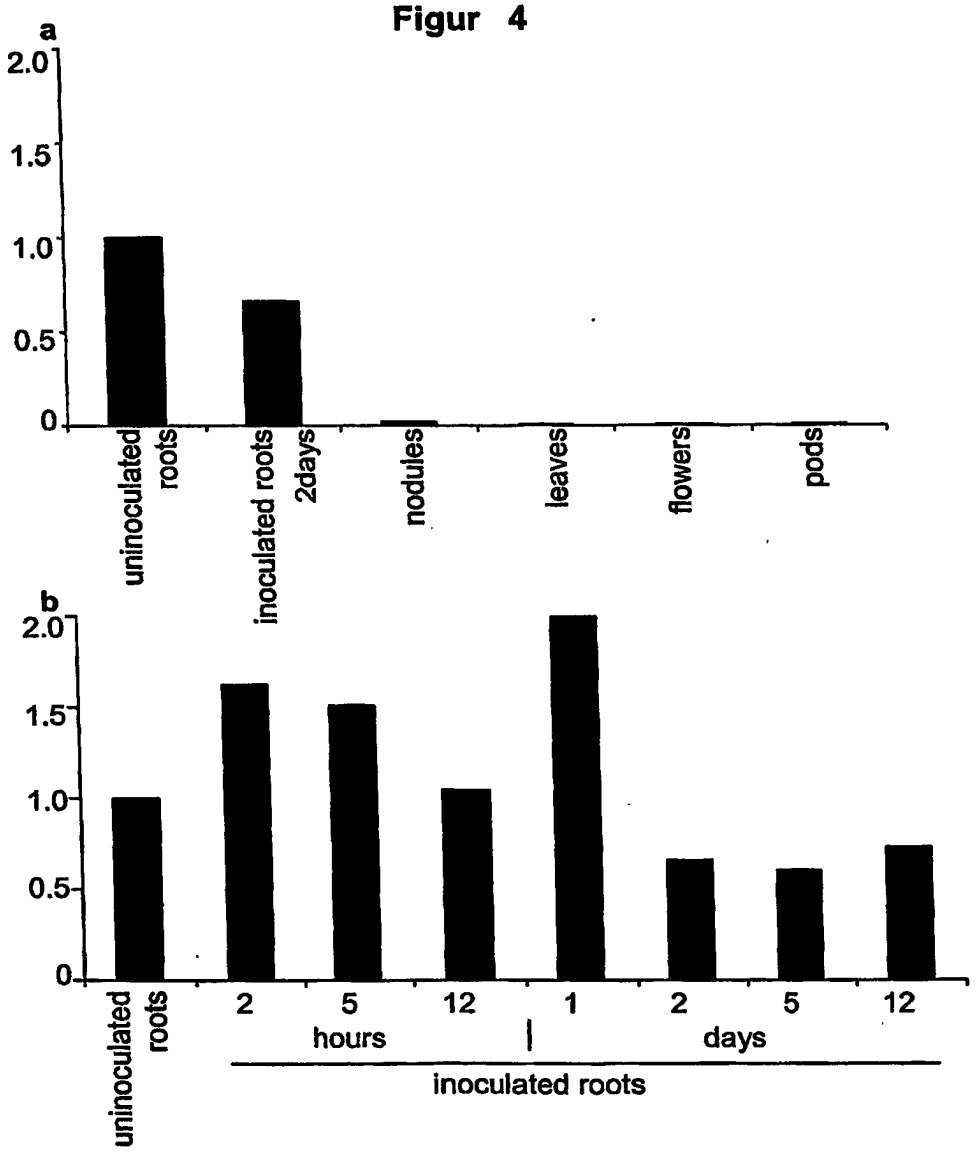
Smart      195:KIADFGLSR..DLYSDDYYKVKGGKLPIRWMAPESLKEGKFTSKSDVWSFG:248
Arab       500:KIANFGVARILDEGDLDLQLTRHVEGTQGYLAPEYVENGVITSKLDVFAFG:550
NFR5       448:KIANFAMARTSTN.............................PMMPKIDVFAFG:472
SYM10      449:KIANFSMARTSTN.............................SMMPKIDVFAFG:473
M.t        450:KIANFGMARTSTN.............................SMMPKIDVFAFG:474
Rice       476:KLSNFSLAKPAAMVD...........................AAATSSDVFAFG:502
```

Figure 3



Inventor: Jensen et al.
Docket No.: 09663.0066USP1
Title: NOD-FACTOR PERCEPTION
Attorney Name: Denise Kettelberger
Phone No.: 612.371.5268
Sheet 4 of 13

Figur 4


a
2.0
1.5
1.0
0.5
0
uninoculated roots
inoculated roots 2days
nodules
leaves
flowers
pods
b
2.0
1.5
1.0
0.5
0
uninoculated roots
2
5
12
1
2
5
12
hours
days
inoculated roots
c
Lotus
leaf
root
nodule
NFR5
Ubiquitin
d
Pisum
nodule
leaf
root
SYM10
Ubiquitin

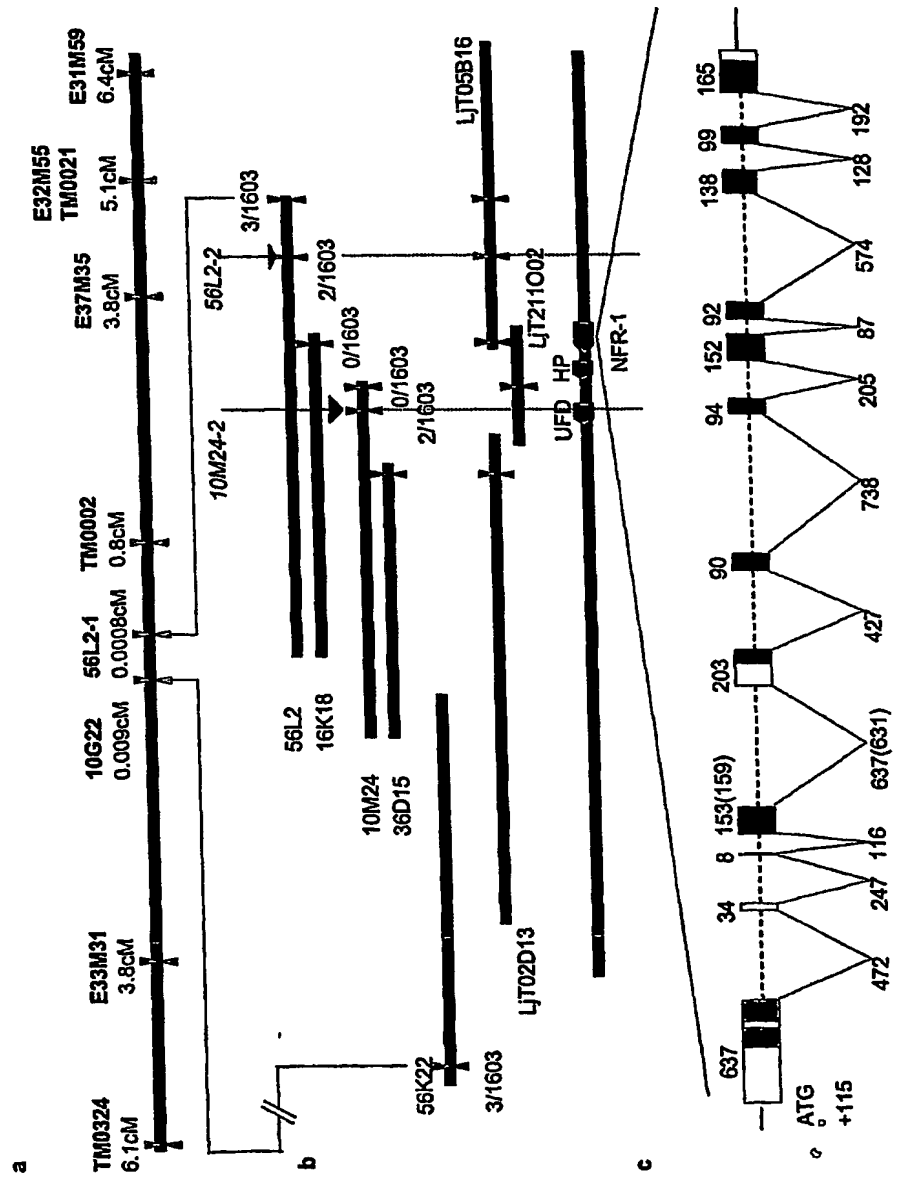

a
TM0324
6.1cM
E33M31
3.8cM
10G22
0.009cM
56L2-1
0.0008cM
TM0002
0.8cM
E37M35
3.8cM
E32M55
TM0021
5.1cM
E31M59
6.4cM
b
56K22
3/1603
10M24
36D15
56L2
16K18
10M24-2
56L2-2
3/1603
2/1603
0/1603
0/1603
2/1603
LjT02D13
LjT05B16
LjT21I002
UFD
HP
NFR-1
c
637
34
8
153(159)
203
90
94
152
92
138
99
165
472
247
116
637(631)
427
738
205
87
574
128
192
ATG
+115

Figure 5

Figure 6

a

```
MKLKTGLLLFFILLLGH                                              SP

VCFHVESNCLKGCDLALASYYILPGVFILQNITTFMQSEIVSSNDAITS
YNKDKILNDINIQSFQRLNIPFPCDCIGGEFLGHVFE                          103

YSASKGDTYETIANLYYANLTTVDLLKRFNSYDPKNIPVNAKVNVT                 LysM1

VNCSCGNSQVSKDYGLFIT                                            168

YPIRPGDTLQDIANQSSLDAGLIQSFNPSVNFSKDSGIAFIP                     LysM2

GRYKNGVYVPLYHR                                                 224

TAGLASGAAVGISIAGTFVLLLLAFCMYV                                  TM

RYQKKEEEKAKLPTDISMALSTQDA(GN)SSSAEYETSGSSGPGTASAT
GLTSIMVAKSMEFSYQELAKATNN                                       322(324)

FSLDNKIGQGGFGAVYYAELRGKKTAIKKMDVQASTEFLCELKVLTHV               KD
         I                  II          III
HHLNLVRLIGYCVEGSLFLVYEHIDNGNLGQYLHGSGKEPLPWSSRVQIA
       IV                 V                        VIa
LDAARGLEYIHEHTVPVYIHRDVKSANILIDKNLRGKVADFGLTKLIEVG
     VIa                  VIb              VII
NSTLQTRLVGTFGYMPPEYAQYGDISPKIDVYAFGVVLFELISAKNAVLKT           621(623)
           VIII     *          IX
GELVAESKGLVALFEEALNKSDPCDALRKLVDPRLGENYPIDSVLKIAQLG
     *
RACTRDNPLLRPSMRSLVVALMTLSSLTEDCDDESSYESQTLINLLSVR*
          XI
```

b

```
SMART0257          YT K  DTLSS ARR GISVS--  LELNNIL  DNLQ GQ  KIP-
NFR1-M1       104  Y AS  DT  T ANL     V  LKRFNSY  KN  N  V  T--149
At21630-M1    105  Y  RQEDT  RVAISN     MESLQARNPFPATN  LS T  LV-151

SMART0257          YTVKK D  SSIARRY I VSDLLE NN-ILD DNLQV QKLK P
NFR1-M2       167  YPIR  D  QDIANQSSLDAGL  SFN -S  FSKDSG-  AF P-208
At21630-M2    170  YPLR EDS SSIARSS V ADIL RYN -G  FNSGNG-  VYVP-211
BAB89226-M2   168  YAVQD D  GNIASLFRS WKD LD N RVA  DFIKP W LF P-212
Volvox M       42  YTIQ  D FWAIAQRR TTVDV  S N -G  ARLQV QVINVP-85
```

Figure 7

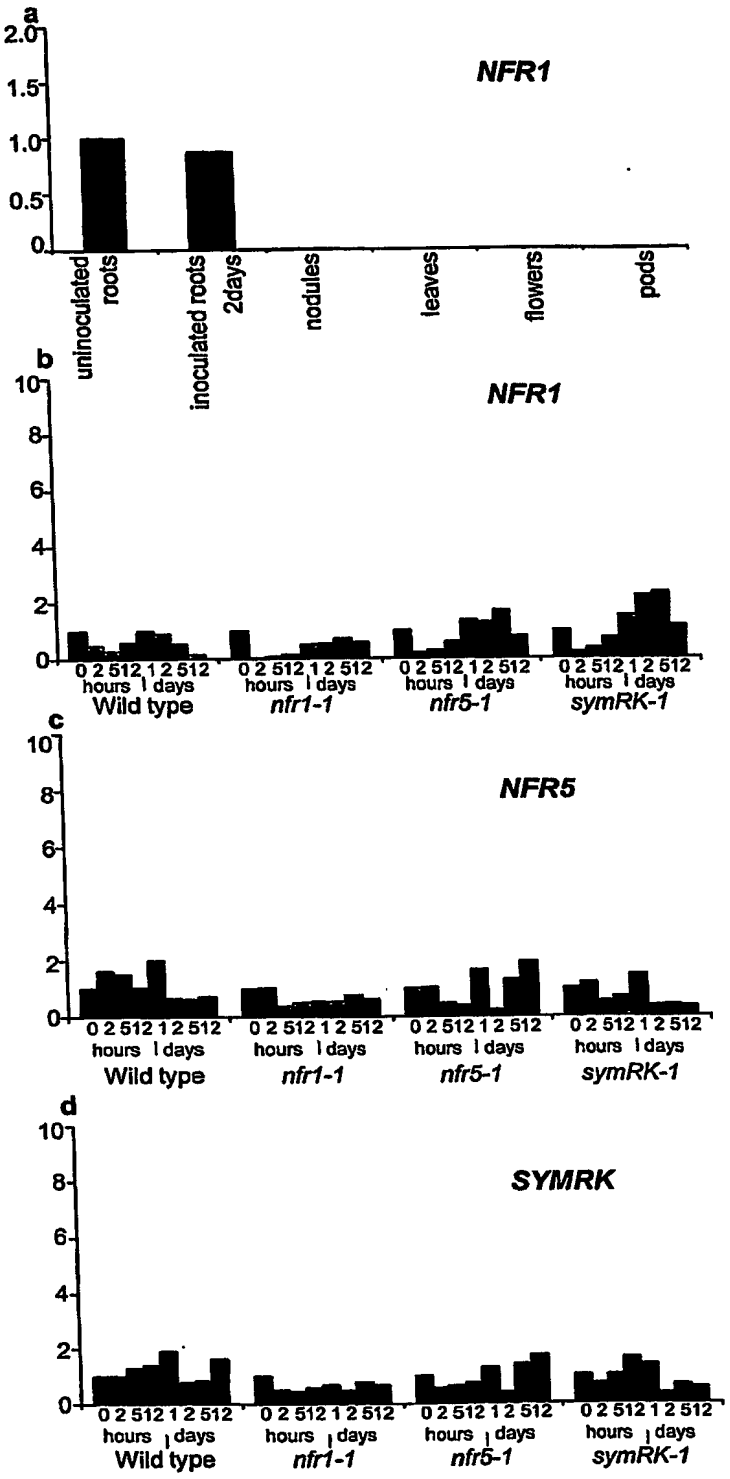

a
NFR1
2.0
1.5
1.0
0.5
0
uninoculated roots
inoculated roots 2days
nodules
leaves
flowers
pods
b
NFR1
10
8
6
4
2
0
0 2 512 1 2 512 hours 1 days
Wild type
nfr1-1
nfr5-1
symRK-1
c
NFR5
d
SYMRK

Figure 8

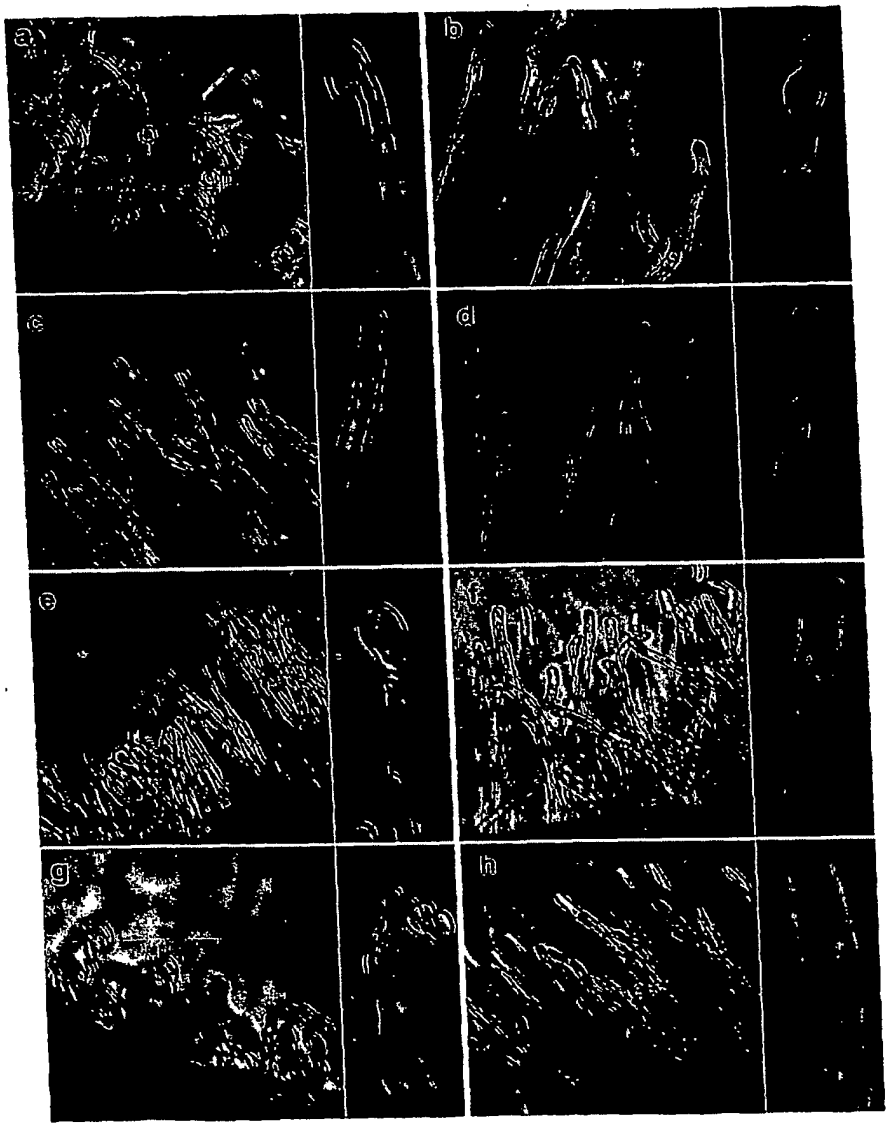
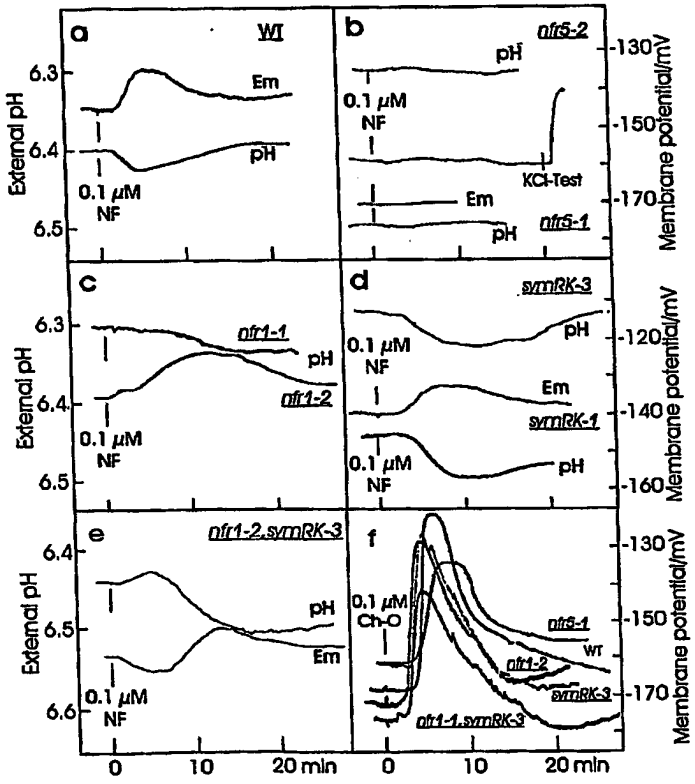


Figure 9


a
WT
Em
pH
0.1 µM
NF
b
nfs-2
pH
0.1 µM
NF
KCl-Test
Em
pH
nfs-1
c
nfr1-1
pH
nfr1-2
0.1 µM
NF
d
symRK-3
pH
0.1 µM
NF
Em
symRK-1
0.1 µM
NF
pH
e
nfr1-2.symRK-3
pH
Em
0.1 µM
NF
f
0.1 µM
Ch-O
nfs-1
WT
nfr1-2
symRK-3
nfr1-1.symRK-3
External pH
6.3
6.4
6.5
6.6
Membrane potential/mV
-130
-150
-170
-120
-140
-160
0
10
20 min

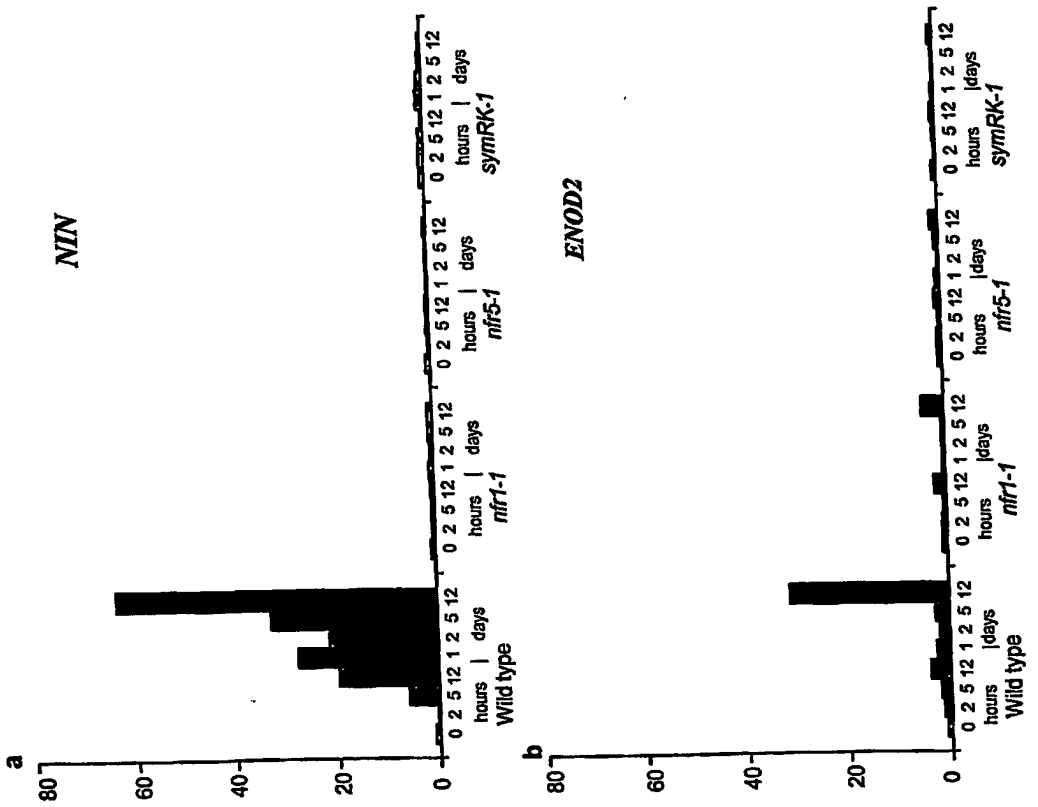

a
NIN
80
60
40
20
0
0 2 5 12 1 2 5 12
hours | days
Wild type
nfr1-1
nfr5-1
symRK-1
b
ENOD2

Figure 10

Figure 11

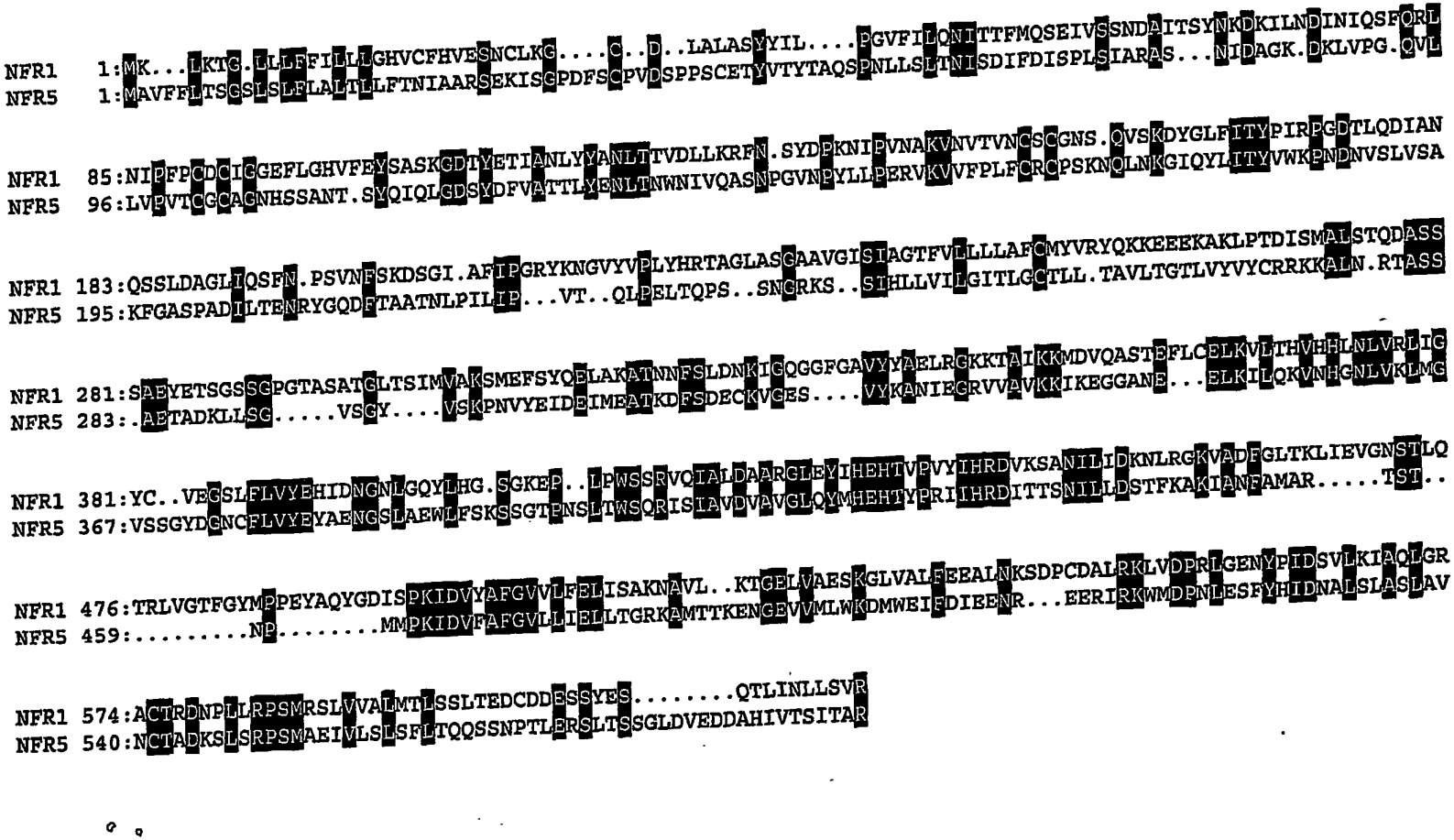


Figure 12

Protein domain structure of *Lotus japonicus* and *Lotus filicaulis* NFR1 and NFR5 proteins and of the hybrid proteins